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(54) Title: GENES FOR ASSESSING CARDIOVASCULAR STATUS AND COMPOSITIONS FOR USE THEREOF

(57) Abstract

The present invention provides methods for assessing cardiovascular status in an individual, which comprise determining the sequence at one or more polymorphic positions within the human genes encoding ACE, AT1, AGT, renin, aldosterone synthase, type–2 angiotensin II receptor, endothelin receptor, and β–adrenoceptor. The invention also provides isolated nucleic acids encoding polymorphisms in genes encoding ACE, AT1, AGT, renin, aldosterone synthase, type–2 angiotensin II receptor, endothelin receptor and β–adrenoceptor, nucleic acid probes that hybridized to polymorphic positions, kits for the prediction of cardiovascular status, and nucleic acid and peptide targets for use in identifying candidate cardiovascular drugs.

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GENES FOR ASSESSING CARDIOVASCULAR STATUS AND COMPOSITIONS FOR USE THEREOF

FIELD OF THE INVENTION

The present invention relates to genetic polymorphisms and polymorphism patterns useful for assessing cardiovascular status in humans. More particularly, the invention relates to identifying and using polymorphism patterns comprising a polymorphism in the a gene encoding a polypeptide selected from the group consisting of ACE, AT1, AGT, renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, and β-adrenoceptor to predict a treatment outcome or likelihood of developing cardiovascular disease, and to assist in diagnosis and in prescription of an effective therapeutic regimen.

BACKGROUND OF THE INVENTION

The renin-angiotensin-aldosterone system (RAAS) plays an important role in cardiovascular physiology in mammals. Specifically, RAAS regulates salt-water homeostasis and the maintenance of vascular tone. Stimulation or inhibition of this system raises or lowers blood pressure, respectively, and disturbances in this system may be involved in the etiology of, for example, hypertension, stroke, and myocardial infarction. The RAAS system may also have other functions such as, *e.g.*, control of cell growth. The renin-angiotensin system includes renin, angiotensin converting enzyme

(ACE), angiotensinogen (AGT), type 1 angiotensin II receptor (AT1), type 2 angiotensin II receptor (AT2) and aldosterone synthase.

International Patent Application No. PCT/IB98/00475, filed April 1, 1998, discloses for the first time an association of polymorphism patterns in ACE, AGT, and AT1 genes with cardiovascular status, particularly with the ability to predict the therapeutic outcome of a particular treatment regimen.

Other genes express polypeptides involved in pathways other than RAAS that also play a role in the regulation of cardiovascular physiology, although it is believed that prior to the present invention, no known association of polymorphism patterns in these genes with cardiovascular status has been observed or reported. Among these other regulators of cardiovascular physiology are endothelin, endothelin receptor, and β-adrenergic receptors 1 and 2.

RAAS Pathway Components

AGT is the specific substrate of renin, an aspartyl protease. The human AGT gene contains five exons and four introns which span 13Kb (Gaillard *et al.*, DNA, 1989, 8:87-99; Fukamizu *et al.*, J. Biol. Chem., 1990, 265:7576-7582). The first exon (37 bp) codes for the 5' untranslated region of the mRNA. The second exon codes for the signal peptide and the first 252 amino acids of the mature protein. Exons 3 and 4 are shorter and code for 90 and 48 amino acids, respectively. Exon 5 contains a short coding sequence (62 amino acids) and the 3'-untranslated region.

Plasma AGT is synthesized primarily in the liver and its expression is positively regulated by estrogens, glucocorticoids, thyroid hormones, and angiotensin II (Ang II) (Clauser *et al.*, Am. J. Hypertension, 1989, 2:403-410). Cleavage of the amino-terminal segment of AGT by renin releases a decapeptide prohormone, angiotensin-I, which is further processed to the active octapeptide angiotensin II by the dipeptidyl carboxypeptidase designated angiotensin-converting enzyme (ACE). Cleavage of AGT by renin is the rate-limiting step in the activation of the renin-angiotensin system.

Several epidemiological observations indicate a possible role of AGT in blood pressure regulation. A highly significant correlation between plasma AGT concentration and blood pressure has been observed in epidemiological studies (Walker *et al.*, J. Hypertension, 1979, 1:287-291). Interestingly, a number of allelic dimorphisms have been identified in the AGT gene. The frequency of at least two of them (174M and 235T) have been partially characterized and in certain populations shown to be

significantly elevated in hypertensive subjects (Jeunemaitre *et al.*, Cell, 1992, 71:169-180). In addition, a specific polymorphism, 235T, has been suggested to be directly involved in coronary atherosclerosis (Ishigami *et al.*, Circulation, 1995, 91:951-4). Futhermore, the presence of A or G at position 1218 in the AGT regulatory region has been correlated with differences in *in vitro* transcriptional capacity for this gene (Inoue et. al., J. Clin. Invest., 1997, 99:1786). However, the foregoing are studies involving only one or at most two polymorphisms. Furthermore, the sole disclosed use is susceptibility to disease.

The human ACE gene is also a candidate as a marker for hypertension and myocardial infarction. ACE inhibitors constitute an important and effective therapeutic approach in the control of human hypertension (Sassaho *et al.*, Am. J. Med., 1987, 83:227-235). In plasma and on the surface of endothelial cells, ACE converts the inactive angiotensin I molecule (Ang I) into active angiotensin II (Ang II) (Bottari *et al.*, Front. Neuroendocrinology, 1993, 14:123-171). Another ACE substrate is bradykinin, a potent vasodilator and inhibitor of smooth muscle cell proliferation, which is inactivated by ACE (Ehlers *et al.*, Biochemistry, 1989, 28:5311-5318; Erdos, E.G., Hypertension, 1990, 16:363-370; Johnston, C.I. *Drugs* (suppl. 1), 1990, 39:21-31).

Levels of ACE are very stable within individuals, but differ greatly between individuals. A greater risk of myocardial infarction has been identified in a group of subjects with an ACE polymorphism designated ACE-DD (Cambien *et al.*, Nature, 1992, 359:641-644), and a 12-fold greater risk of myocardial infarction has been identified in a subgroup of patients having a combination of the ACE polymorphism ACE-DD and one of the AGT polymorphisms (235T) described above (Kamitani *et al.*, Hypertension, 1994, 24:381). Recently, six ACE polymorphisms were identified and characterized (Villard *et al.*, Am. J. Human Genet., 1996, 58:1268-1278).

The vasoconstrictive, cell growth-promoting and salt conserving actions of angiotensin II are mediated through binding to and activation of angiotensin receptors, of which at least two types have been cloned (AT1 and AT2). The type 1 Ang II receptor (AT1), a G-protein-coupled seven transmembrane domain protein, is widely distributed in the body and mediates almost all known Ang II effects (Fyhrquist *et al.*, J. Hum. Hypertension, 1995, 5:519-524).

Several polymorphisms have been identified in the AT1 receptor gene. Initial studies suggest that at least one of them is more frequent in hypertensive subjects

($AT^{1166}C$)(Bonnardeaux *et al.*, Hypertension, 1994, 24:63-69). This polymorphism, combined with the ACE-DD polymorphism, has been shown to correlate strongly with the risk of myocardial infarction (Tiret *et al.*, Lancet, 1994, 344:910-913).

Endothelin (ET) Regulation of Cardiovascular Physiology

Endothelin is a potent vasoconstrictive peptide characterized by long lasting action. It was first discovered as a vasoconstricting factor in conditioned medium (Hickey *et al.*, Am. J. Physiol., 1985, 248:C550), and subsequently purified and characterized (Yanagisawa *et al.*, Nature, 1988, 332:411). ET is produced as preproendothelin, which is cleaved after removal of the signal sequence by an endopeptidase, followed by cleavage with endothelin converting enzyme (Xu *et al.*, Cell, 1994, 78:473; Shimada *et al.*, J. Biol. Chem., 1994, 269:18274). Analysis of the human *ET* gene has revealed the existence of two additional ET-like peptides expressed in various tissues, termed ET-2 and ET-3 (Inoue *et al.*, Proc. Natl. Acad. Sci. USA, 1989, 86:2863). The first endothelin was accordingly termed ET-1.

One of the important discoveries following characterization of ETs was the discovery of two ET receptors, ET_A and ET_B (Arai *et al.*, Nature, 1990, 348:730; Sakumi *et al.*, Nature, 1990, 348:782). Both belong to the family of heptahelical G-protein coupled receptors. There is 68% amino acid identity between the two receptor subtypes. ET_A exists as a single copy gene located on human chromosome 4 (Hosoda *et al.*, J. Biol. Chem., 1992, 267:18797; Cyr *et al.*, Biochem. Biophys. Res. Commun., 1991, 181:184). ET_B exists as a single copy gene located on human chromosome 13 (Arai *et al.*, J. Biol. Chem., 1993, 268:3463-70), although a splice variant of ET_B has been found (Shyamala *et al.*, Cell. Mol. Biol. Res., 1994, 40:285-96). The cDNA sequence of ET_A has been deposited with GenBank with accession number S57498.

In the early stage of ET research, a great number of pharmacological studies suggested that the responses to ETs could be divided into two groups according to the pharmacological potency of the three peptides. Indeed, these two receptors, ET_A and ET_B, are distinct in their ligand binding affinity and distribution in tissues and cells. ET_A has a high affinity to ET-1 and ET-2, but a low affinity to ET-3. ET_B has equally potent affinities to all three endogenous ETs. ET_A exists on smooth muscle and mediates vasoconstriction. In contrast, ET_B exists on endothelium and mediates the release of

relaxing factors such as nitric oxide and prostacycline. However, several reports demonstrated that ET_B on some vascular smooth muscle also mediated vasoconstriction.

Cloning of the *ET receptor* gene facilitated the development of ET-receptor antagonists, such as BE-18257B, and BQ-123 and FR139317, two derivatives of BE-18257B (See Masaki, Cardiovascular Res., 1998, 39:530). Many selective and non-selective antagonists for ET_A and ET_B have emerged.

Although the pathophysiological role of ET is still unclear, ET antagonists demonstrated significant beneficial effects in pathological conditions, including congestive heart failure, pulmonary hypertension, cerebrovascular spasm after subarachnoid hemorrhage, acute renal failure, and essential hypertension. ET or ET receptor knockout mice have also provided important information regarding the physiological and pathophysiological significance of ET (Masaki, *supra*). In particular, mice with a knockout of *ET-3* or the *ET_B receptor* genes exhibit phenotypic changes that resemble Hirschsprung's disease, a human hereditary syndrome associated with a missense mutation of the *ET_B* gene (Pfiffenberger, *et al.*, Cell, 1994, 79:1257).

Despite advances in understanding the role of the endothelin pathway in the treatment of cardiovascular diseases, questions remain. Not all experimental models of hypertension respond to endothelin antagonists, and it remains a uncertain whether endothelin antagonists improve cardiac structure and function beyond the benefits of blood pressure reduction (Moreau, Cardiovascular Res., 1998, 39:534). Thus, there is a need in the art for a reliable and effective means for predicting whether endothelin antagonists will be effective for treating hypertension in a given individual.

β-Adrenergic Receptors (β-Adrenoceptors)

The adrenoceptors fall into three major groups, α_1, α_2, β, within each of which further subtypes can be distinguished pharmacologically (Lüllmann, *et al.* in Color Atlas of Pharmacology, New York, 1993). Adrenergic receptors are all G-protein linked. They are involved in regulation of the cardiovascular system, and in the control of metabolic activity, *e.g.*, insulin secretion and glucose release. They also mediate constriction or relaxation of smooth muscle cells in the respiratory, gastrointestinal, and genitourinary tracts (Berne and Levy, Principles of Physiology (2nd Ed.), Mosby-Year Books, Inc., 1996, pp. 691-696).

Adrenoceptors are targets for epinephrine and norepinephrine, which are representatives of the family of monoamine neurotransmitters. Epinephrine has equally high affinity for all α- and β-receptors while norepinephrine differs from epinephrine by its low affinity for β_2-receptors (The Biochemical Basis of Neuropharmacology, (7th Ed.) New York, 1996, pp. 226-292). The adrenoceptors themselves interact preferentially with three different classes of G-proteins: G_S (β-adrenoceptors) mediating activation of adenylate cyclase, G_i (α_2-adrenoceptors) mediating inhibition of adenylate cyclase, and G_q (α_1-adrenoceptors) mediating activation of phospholipase C (Hieble *et al.*, J. of Med. Chem., 1995, 38:3415-3444).

The pharmacological interest of adrenoceptors is mainly for the treatment of cardiovascular diseases, *e.g.*. through the development of β-antagonists, α_1-antagonists and α_2-agonists to treat hypertension, but they are also considered important for the treatment of asthma (β_2-agonists).

The β_2-adrenergic receptor is expressed on a number of cell types, *e.g.*, bronchial smooth muscle, where its activation results in relaxation and bronchial dilatation. These receptors are also being expressed on epithelial cells, vascular endothelium, alveolar walls, immune cells, and presynaptic nerve terminals (Liggett, Am. J. Respir. Crit. Care. Med., 1997, 156:S156-S162). Cardiac cells express mainly β_1-, but also a small fraction of β_2-adrenoceptors (Collins *et al.*, Biochimica et Biophysica Acta, 1993, 1172:171-174). β_1-adrenoceptors are also expressed in brain and pineal gland.

β-Adrenoceptor Function

The β_1- and β_2-adrenergic receptors are coupled to a G_S-protein complex, which activates adenylate cyclase. Agonist binding to the β_1-receptor, located in cardiac muscle cells, mediates increased contractility and cardiac output (Lüllmann, *supra*). Agonist binding to the β_2-receptor, located in peripheral vascular arteries, mediates vasodilation by increasing the amount of cAMP, and thereby inhibiting activation of myosin kinase, which is necessary for smooth muscle cell constriction (*ibid.*) Activation of cAMP in cardiac cells by agonist binding to both β_1- and β-$_2$-adrenoceptors activates the cAMP-dependent protein kinase (PKA) (Castellano and Böhm, Hypertension, 1997, 29:715-722). β-adrenoceptors also regulate the control of melatonin production in the pineal gland, by the cAMP activation of one of the enzymes (5-HT-N-acetyl transferase) involved in the synthesis of melatonin (Collins *et al.*, *supra*).

The β_2-receptors mediate increased conversion of glycogen to glucose (glycogenolysis) in both the liver and skeletal muscle (H. Lüllmann, *et al.*, *supra*), and stimulate influx of potassium into muscle cells to prevent hyperkalemia (Berne and Levy, *supra*).

The β-receptors are regulated on the protein level by desensitization. The initial desensitization process results from the phosphorylation of serine and threonine residues in the cytoplasmic tail or third intracellular loop by several protein kinases, including βARK and PKA (Hieble *et al.*, *supra*). βARK phosphorylates specific serine or threonine residues in the C-terminal of receptors that are occupied by an agonist. The phosphorylation triggers binding of the cytostolic protein β-arrestin and results in the uncoupling from $G_{s\alpha}$. PKA is activated by cAMP and phosphorylates the β_2-adrenoceptor by a relatively slow process. The phosphorylated receptor loses the ability to activate G_s (Castellano and Böhm, *supra*). Prolonged interaction of agonists with adrenoceptors generally results in receptor desensitization.

β-Adrenoceptor Gene Structure

The human β_1-adrenoceptor gene is located on the long arm of chromosome 10, the same chromosome as for the α_{2A}-adrenoceptor gene. The coding sequence of this gene is deposited with GenBank, accession number X69168. The regulatory region is also deposited with GenBank, accession number J03019. It codes for an intronless gene product of 1431 base pairs (Hall, Thorax, 1996, 51:351-353). Both the promoter and the coding region of the gene are rich in G and C residues, which make up greater than 70% of the bases. The promoter does not contain any paired consensus TATA box and CAAT box elements but instead clusters with an inverted CAAT box and SP_1 or AP-2 binding motifs. This type of receptor, reminiscent of "housekeeping genes", has been described for other G-protein coupled receptors as well (Collins *et al.*, *supra*).

The human β_2-adrenoceptor gene is located on the long arm of chromosome 5, the same chromosome as the α_{1B}-adrenoceptor gene. The coding sequence has been deposited with GenBank, with accession numbers M15169, J02728, or M16106. The regulatory region sequence is also deposited with GenBank, accession number Y00106. It codes for an intronless gene product of 1239 base pairs (Hall, *supra*). The promoter region is 200-300 bases 5' of the translation initiation codon, and it can form strong secondary structures due to high G-C content. There are two TATA boxes (separated by roughly 10

bp) and a CAAT box located approximately 30 and 80 base pairs upstream, respectively, from the mRNA start region.

β-Adrenoceptor Gene Regulation

There are some regulatory regions identified in the promoter region of the β_1-adrenoceptor gene: a cAMP response element (CRE), a consensus thyroid response element (TRE), and a glucocorticoid response element (GRE). This is consistent with the evidence that both thyroid hormone and corticosteroids affect adrenergic sensitivity in both heart and adipose tissue. The CRE region might have a self-regulatory function, as has been shown for the β_2-adrenoceptor gene (Collins et al., *supra*).

There are several regulatory domains in the 5' flanking region of the β_2-adrenoceptor. Among these is a cAMP-responsive element (CRE), which is recognized and stimulated by a phosphoprotein called CRE binding protein (CREB). CREB is partially under the control of PKA-dependent phosphorylation processes. This is seen as an increase in β_2-adrenoceptor mRNA level in the early phase after exposure to β-agonists. However, the level of mRNA is decreased after prolonged exposure to agonists, probably mediated by a shortening of mRNA half-life (Castellano and Böhm, *supra*). It has also been shown that transcription of the β_2-adrenoceptor gene is upregulated by stimulation with glucocorticoids in a variety of tissues (Collins et al., *supra*). In the 3' flanking region there are sequences homologous to glucocorticoid response elements. These might be responsible for the increased expression of β_2 adrenoceptor observed in transfected cells after treatment with hydrocortisone (Emorine and Marullo, Proc. Natl. Acad. Sci. USA, 1987, 84:6995-6999).

β-Adrenoceptor Protein Structure

The proposed model for β-adrenoceptors is like most of the G-protein binding receptors, a seven α-helical transmembrane structure, where the seven α-helices are radially arranged around a central "pore", in which the receptor ligands bind. The β-adrenoceptors have an extracellular glycocylated N-terminus, and an intracellular C-terminus. The β_1-receptor consists of 477 amino acids; the β_2-receptor consists of 413 amino acids.

The overall amino acid identity of human β_1- and β_2- adrenoceptors is only 54%. However, it is likely that the pharmacological differences between β_2-receptors and

β_1-receptors are due to subtle changes in orientation of the primary binding sites, resulting in a slightly different binding site rather than to specific amino acid substitutions (Hieble *et al.*, J. Med. Chem. 1995, 38:3415-3444).

Site-directed mutagenesis has demonstrated that an aspartic acid residue, Asp-113, located in the third transmembrane-spanning helix, and two serine residues, Ser-204 and Ser-207, are required for full agonist binding to the β_2-adrenoceptor. The β_1-adrenoceptor contains identical amino acid residues located in corresponding positions to those shown to be important for agonist binding to the β_2-adrenoceptor. Another aspartic acid residue, Asp-79, located in the second α-helix of both β-receptors is highly conserved in G-protein coupled receptors (Hieble *et al.*, *supra*). Ser-319 has a potential role in agonist binding to the β_2-adrenoceptor.

Mutation of Tyr-350, located in the cytoplasmic tail of the β_2-receptor, interferes with coupling of the receptor to G_S (Hieble *et al.*, *supra*). Also, palmitoylation of Cys-341 in the C-terminal enables the β_2-adrenoceptor to form a fourth intracytoplasmic loop, which increases the ability of the agonist-bound receptor to mediate adenylyl cyclase stimulation (Strosberg, Preotein Science, 1993, 2:1198-1209).

β-Adrenoceptors as Drug Targets

No cause of disease can be identified in 80-90% of patients with hypertension. They have so-called essential hypertension, which affects 5-10% of the general population, and is the most common cause of disease in developed countries (J. Axford, *Medicine*, Blackwell Science Ltd., 1996, 10.119-10.130). Betablockers have been widely used in the treatment of hypertension. They are particularly useful for the treatment of juvenile hypertension with tachycardia and high cardiac output. Betablockers or beta-adrenergic blockers were first introduced as a treatment for essential hypertension in 1964, and are still recommended as first choice because the cost for betablockers is low, which improves patient compliance. They act by binding to β_1-receptors on the cardiac smooth muscle cells, which leads to decreased cardiac output. Most betablockers are not specific β_1-receptor antagonists but binds to β_2-receptors as well. The binding to β_2-receptors gives the opposite of the desired effect though inhibition of β_2-receptors leads to vasoconstriction. This gives a side effect with cold hands and feet because most of the β_2-receptors are located in the peripheral vascular arteries.

They have also been known to cause bronchospasms as well as some central nervous system side effects (nightmares, somnolence). They decrease insulin secretion, which makes them inappropriate to treat hypertensives with diabetes mellitus, and they can cause heart failure and peripheral artery obstructive disease (Velaseco and Rodrigues, Journal of Human Hypertension 10, Suppl. 1, S77-S80, 1996).

β-adrenoceptor agonists, such as dopamine and dobutamine are used to stimulate myocardial β_1-adrenoceptors in the acute management of congestive heart failure. They act by increasing contractility and cardiac output.

A lot of different β_2-agonists are used in the treatment of asthma. They exert their primary effect on the β_2-adrenergic receptor of bronchial smooth muscle, resulting in relaxation and bronchial dilatation. They also protect against bronchoconstrictor challenge (Hall, *supra*).

Thus, there is a clear need in the art for an improved understanding of the effects of betablockers on different subjects, and to predict which patients will have a better response to treatment with betablockers.

Need for Effective Cardiovascular Status Assessment

The high morbidity and mortality associated with cardiovascular disease demonstrate a need in the art for methods and compositions that allow the determination and/or prediction of the therapeutic regimen that will result in the most effective treatment outcome in a patient suffering from cardiovascular disease. This includes identification of individuals who are more or less responsive to particular therapeutic regimens, including, *e.g.*, particular drugs that are conventionally used to treat cardiovascular disease.

Furthermore, the heterogeneity in responses to cardiovascular therapies emphasizes a need for another approach to rational drug development. In particular, populations that are identified as non-responsive to a particular therapeutic regimen can be identified for development and testing of alternative regimens. Thus, effective treatment regimens could be developed for a larger percentage of the affected population.

In summary, there is a need to reduce or eliminate trial and error in selecting a therapeutic regimen for a particular individual. It would be desirable instead to predict whether a given individual will be responsive to, *e.g.*, a particular class of drugs or even to a particular drug or whether he/she is likely to suffer from adverse reactions or side-effects.

There is also a need in the art for methods and compositions that allow the identification of individuals having a predisposition to cardiovascular disease, such as, *e.g.*, myocardial infarction, hypertension, atherosclerosis, and stroke, to facilitate early intervention and disease prevention.

The present invention addresses these and other needs in the art by providing polymorphisms and polymorphic patterns that are characteristic of cardiovascular status, and by using these polymorphisms and patterns to prescribe or to develop more effective treatments or to assist in diagnosis.

Citation of any reference in this application should not be construed as an admission that the reference is prior art to the invention. Each cited reference is incorporated herein by reference in its entirety.

SUMMARY OF THE INVENTION

In one aspect, the present invention provides reagents and methods for predicting whether a particular therapeutic regime (such as a specific drug, a class of drugs or any other therapeutic regime, pharmacological or not) would be effective in improving a cardiovascular condition in a human individual, or would be ineffective for that purpose, or its use would be associated with adverse reactions or undesirable side-effects.

A particular advantage of the invention is that one or more polymorphic markers provide a basis for predicting the outcome of a treatment regimen. By comparing a polymorphic pattern of a subject who requires treatment for a cardiovascular disorder, for example hypertension, with a reference pattern previously established to correlate with responsivity to the treatment regimen, a physician can predict whether a treatment plan, such as administration of an ACE inhibitor, is likely or not to be effective before subjecting the subject to the treatment plan. For example, a comparison of the test polymorphic pattern from an individual with reference polymorphic patterns of individuals exhibiting differing responses to a particular therapeutic intervention can be used to predict the type or degree of responsivity of the individual to such intervention. The present invention thus represents a significant breakthrough in treating cardiovascular pathologies in that it reduces or eliminates trial and error in selecting a treatment for a particular individual cardiovascular patient.

An additional advantage of the invention derives from the ability to eliminate subjects from clinical trials who are predictably non-responsive, or at risk for an

adverse response, to a particular treatment regimen. Furthermore, adverse results in an early trial can be evaluated to identify polymorphic patterns, so that the adverse results can be correlated with a sub-population of the test population permitting exclusion of such sub-population from the treatment group. The invention may thus ensure that a beneficial drug can be approved for use in the appropriate population, and decrease the number of required patients and therefore the duration and cost of clinical trials. It may also lead to identification of another subgroup which can be the target for development of another therapeutic regimen.

All of the foregoing applications within the scope of the invention can be deemed to be assessments of an individual's cardiovascular status, as the term is broadly defined below.

The foregoing methods of the invention are carried out by comparing a test polymorphic pattern established by at least one polymorphic position within a gene encoding ACE, AT1, AGT, renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, and β-adrenoceptor with a polymorphic pattern of a population of individuals exhibiting a predetermined responsivity to the regimen (reference pattern). If the test pattern matches the reference pattern, there is a statistically significant probability that the individual has the same cardiovascular status as that correlated with the reference pattern.

The polymorphic pattern preferably consists of at least two (and more preferably at least three) polymorphic positions, at least one of which is in the gene encoding a polypeptide from the group consisting of ACE, AT1, AGT, renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, and β-adrenoceptor, and a second polymorphism in a gene encoding a polypeptide selected from the group consisting of ACE, AT1, AGT, renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, and β-adrenoceptor.

Additionally, the invention provides methods for assessing whether a particular individual has a genetic predisposition to a cardiovascular pathology. This aspect of the invention comprises comparing a test polymorphic pattern established by at least one and preferably at least two and most preferably at least three polymorphic positions within a gene encoding ACE, AT1, AGT, renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, and β-adrenoceptor in conjunction with one or more, and preferably two or more, other polymorphic positions in ACE, AT1, AGT, renin,

aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, or β-adrenoceptor, with a polymorphic pattern of individuals exhibiting a predisposition to a cardiovascular syndrome. The conclusion drawn depends on whether the individual's polyymorphism pattern matches the reference pattern.

The invention also provides an isolated nucleic acid having a sequence corresponding to part or all of the gene encoding ACE, AT1, AGT, renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, and β-adrenoceptor, the nucleic acid comprising a polymorphism in the ACE, AT1, AGT, renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, and β-adrenoceptor gene. In preferred embodiments, the polymorphism, in combination with one or more other polymorphisms in the sequence of the same gene or a gene encoding a protein selected from the group consisting of human ACE, AT1, AGT, renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, and β-adrenoceptor, is predictive of a particular type or level of responsivity to a given treatment, or indicates a predisposition to one or more clinical syndromes associated with cardiovascular disease, or both.

The isolated polymorphisms according to the invention (which are described using the numbering indicated in Table 1 below) include without limitation:

Nucleic acids encoding renin having one or more polymorphic positions in the last exon of the gene (exon 10), which is a cytosine to thymine transition that creates a premature stop codon at position 387.

Nucleic acids encoding aldosterone synthase promoter at position -344.

Nucleic acids encoding β-adrenergic receptor-1 regulator region, at positions 2238, 2440, 2493, 2502, 2577, 2585, 2693, 2724, and 2757, as numbered in GenBank accession number X69168. In preferred embodiments, the bases at specific positions are 2238 G, 2238 A, 2577 C, 2257 T, 2757 A, and 2757 G.

Nucleic acids encoding β-adrenergic receptor-1 coding region, at positions 231, 758, 1037, 1251, 1403, and 1528, as numbered in GenBank accession number J03019. In preferred embodiments, the bases at specific positions are 231 A, 231 G, 1251 C, 1251 G, 1403 A, 1403 G, 1528 C, and 1528 A.

Nucleic acids encoding β-adrenergic receptor-2 regulatory region, at positions 932, 934, 987, 1006, 1120, 1221, 1541, and 1568, as numbered in GenBank accession number M15169, or J02728, or M16106. In preferred embodiments, the bases

at specific positions are 934 A, 934 G, 987 C, 987 G, 1006 A, 1006 G, 1120 C, 1120 G, 1221 C, 1221 T, 1541 C, 1541 T, 1568 C, and 1568 T..

Nucleic acids encoding β-adrenergic receptor-2 coding region, at positions 839, 872, 1045, 1284, 1316, 1846, 1891, 2032, 2068, and 2070, as numbered in GenBank accession number Y00106. In preferred embodiments, the bases at specific positions are 839 A, 839 G, 872 C, 872 G, 1045A, 1045 G, 1284 C, 1284 T, 1316 A, 1316 C, 1846 C, 1846 G, 2032 A, 2032 G, 2068 no insert, 2068 G, 2068 C, 2070 no insert, and 2070 C.

Nucleic acids encoding endothelin-A receptor at positions 969, 1005, 1146, and 2485, as numbered in GenBank accession number S57498. In preferred embodiments, the bases at specific positions are 969 C, 969 T, 1005 A, 1005 G, 1146 A, 1146 G, 2485 T, and 2485 C.

Nucleic acids comprising polymorphisms present in other genes, which can be used in combination with a polymorphism from a gene encoding a polypeptide selected from the group consisting of ACE, AT1, AGT, renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, and β-adrenoceptor to establish a polymorhism pattern, have been disclosed in International Patent Application No. PCT/IB98/00475, and include:

(i) Nucleic acids encoding ACE having one or more polymorphic positions at the position in the regulatory region numbered 5106; positions in the coding region numbered 375, 582, 731, 1060, 2741, 3132, 3387, 3503, and 3906; and position 1451 all positions as numbered in GenBank entry X62855. In preferred embodiments, the sequences at the polymorphic positions in the ACE regulatory region are one or more of 5106C and 5106T; and the sequences at the polymorphic positions in the coding region are one or more of 375A, 375C, 582C, 582T, 731A, 731G, 1060G, 1060A, 2741G, 2741T, 3132C, 3132T, 3387T, 3387C, 3503G, 3503C, 3906G, and 3906A. The invention also encompasses a nucleic acid encoding a deletion of nucleotides 1451-1783 as numbered in GenBank entry X62855.

(ii) Nucleic acids encoding AGT having one or more polymorphic positions at positions in the regulatory region numbered 395, 412, 432, 449, 692, 839, 1007, 1072, and 1204; positions in the coding region numbered 273, 912, 997, 1116, and 1174; and position 49 as numbered in GenBank entry M24688. In preferred embodiments, the sequences at the polymorphic positions in the AGT regulatory region are one or more of 395T, 395A, 412C, 412T, 432G, 432A, 449T, 449C, 692C, 692T, 839G, 839A, 1007G,

1007A, 1072G, 1072A, 1204C, and 1204A; the sequences at the polymorphic position in the coding region are one or more of 273C, 273T, 912C, 912T, 997G, 997C, 1116G, 1116A, 1174C and 1174A; and the sequence at position 49 in GenBank entry M24688 is either A or G.

(iii) Nucleic acids encoding AT1 having one or more polymorphic positions at positions in the regulatory region numbered 1427, 1756, 1853, 2046, 2354, 2355, and 2415; and the position in the coding region numbered 449. In preferred embodiments, the sequences at the polymorphic positions in the AT1 regulatory region are one or more of 1427A, 1427T, 1756T, 1756A, 1853T, 1853G, 2046T, 2046C, 2354A, 2354C, 2355G, 2355C, 2415A and 2415G; and the sequences at the polymorphic positions in the coding region are one or more of 449G, 449C, 678T, 678C, 1167A, 1167G, 1271A, and 1271C.

The invention also encompasses libraries of isolated nucleic acid sequences, such as arrays on a solid surface, wherein each sequence in the library comprises a polymorphic position in the gene encoding ACE, AT1, AGT, renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, or β-adrenoceptor and other polymorphic positions in the other genes, including without limitation the polymorphic positions and sequences disclosed herein. Also provided are nucleic acid probes that hybridize specifically to the identified polymorphic positions; peptides and polypeptides comprising polymorphic positions; and polymorphism-specific antibodies, *i.e.*, sequence-specific antibodies that bind differentially to polymorphic variants of the foregoing genes, that can be used to identify particular polymorphic variants.

In another aspect, the invention provides kits for the determination of polymorphic patterns in an individual's genes. The kits comprise a means for detecting polymorphic sequences, including without limitation oligonucleotide probes that hybridize at or adjacent to the polymorphic positions and polymorphism-specific antibodies.

In yet another aspect, the invention provides nucleic acid and polypeptide targets for use in screening methods to identify candidate cardiovascular drugs. Nucleic acid targets may be, *e.g.*, DNA or RNA and are preferably at least about 10, and most preferably at least about 15, residues in length and comprise one or more polymorphic positions in a gene encoding a polypeptide from the group consisting of ACE, AT1, AGT, renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, and β-

adrenoceptor. Peptide targets are at least about 5 amino acids in length and may be as large or larger than the full-length polypeptides.

DETAILED DESCRIPTION OF THE INVENTION

The invention in based, in part, on the discovery that polymorphisms in certain genes in the RAAS, endothelin, and β-adrenoceptors pathways define polymorphism patterns that correlate with cardiovascular status. Most significantly, by comparing a test individual's polymorphism pattern with a reference polymorphism pattern, which is a polymorphism pattern from a population of individuals with known cardiovascular status, one is able to predict whether the test individual has an increased likelihood to share the same cardiovascular status as that correlated with the reference polymorphism pattern. In particular, particular patterns correlate with responsiveness to ACE inhibitors, non-responsiveness to ACE inhibitors, and predisposition to cardiovascular diseases or dysfunctions, including myocardial infarction and stroke.

The invention provides a powerful predictive tool for clinical testing and treatment of cardiovascular disease. For clinical testing, the present invention permits smaller, more efficient clinical trials by identifying individuals who are likely to respond poorly to a treatment regimen and reducing the amount of uninterpretable data. By evaluating a test individual's polymorphism pattern, a physician can prescribe a prophylactic or therapeutic regimen customized to that individual's cardiac status. Adverse responses to particular therapies can be avoided by excluding those individuals whose cardiovascular status puts them at risk for that therapy. Appropriate changes in lifestyle, including diet, environmental stress, and exercise levels can be prescribed for individuals whose test polymorphic pattern matches a reference pattern that correlates with increased predisposition to cardiovascular disease.

Definitions

"Cardiovascular status" as used herein refers to the physiological status of an individual's cardiovascular system, as reflected in one or more status markers or indicators including genotype. Cardiovascular status shall be deemed to include without limitation not only the absence or presence of a pathology or disease in one or more components of the individual's cardiovascular system and the individual's predisposition

to developing such a condition, but also the individual's responsivity, *i.e.*, the ability or inability of the individual to respond (positively or negatively) to a particular prophylactic or therapeutic regimen or treatment for a cardiovascular condition, such as a drug or a class of drugs. A negative response includes one or more adverse reactions and side effects. Status markers include without limitation clinical measurements such as, *e.g.*, blood pressure, electrocardiographic profile, differentiated blood flow analysis, and the presence of increased levels of cellular proteins associated with a cardiovascular event. Examples of such proteins, also called diagnostic markers, which are important in cardiac events include myosin light chain, myosin heavy chain, myoglobin, troponin I, troponin T, CK-MB, etc. (see U.S. Patents No. 5,604,105 and No. 5,744,358). Status markers according to the invention are assessed using conventional methods well known in the art. Also included in the evaluation of cardiovascular status are quantitative or qualitative changes in status markers with time, such as would be used, *e.g.*, in the determination of an individual's response to a particular therapeutic regimen or of a predisposed individual's eventual development of a cardiovascular condition.

Examples of cardiovascular syndromes that are included in the foregoing definition of cardiovascular status include diagnosis of, or predisposition to, one or more cardiovascular syndromes, such as, *e.g.*, hypertension, acute myocardial infarction, silent myocardial infarction, unstable angina, stroke, and atherosclerosis. It will be understood that a diagnosis of a cardiovascular syndrome made by a medical practitioner encompasses not only clinical measurements but also medical judgment.

"Responsivity", as used herein, refers to the type and degree of response an individual exhibits to a particular therapeutic regimen, *i.e.*, the effect of a treatment on an individual. Responsivity breaks down into three major categories: therapeutic effect; no effect; and adverse effect. Naturally, there can be differing degrees of a therapeutic effect, *e.g.*, between full elimination and partial elimination of symptomology. In addition, adverse effects, or side effects, may be observed even though the treatment is beneficial, *i.e.*, therapeutically effective. Indeed, the present invention may permit identification of individuals with complex responsivity traits or patterns.

A "predisposition to develop a cardiovascular syndrome" refers to an increased likelihood, relative to the general population, to develop a cardiovascular syndrome, as defined above. A predisposition does not signify certainty, and development of the syndrome may be forestalled or prevented by prophylaxis, *e.g.*, adopting a modified

diet, exercise program, or treatment with gene therapy or pharmaceuticals. Naturally, an advantage of the present invention is that it permits identification of individuals who are, based on their genotype, predisposed to develop a cardiovascular syndrome, and for whom prophylactic intervention can be especially important.

A "polymorphism" as used herein denotes a variation in the nucleotide sequence of a gene in an individual. Genes that have different nucleotide sequences as a result of a polymorphism are "alleles". A "polymorphic position" is a predetermined nucleotide position within the sequence. In some cases, genetic polymorphisms are reflected by an amino acid sequence variation, and thus a polymorphic position can result in location of a polymorphism in the amino acid sequence at a predetermined position in the sequence of a polypeptide. An individual "homozygous" for a particular polymorphism is one in which both copies of the gene contain the same sequence at the polymorphic position. An individual "heterozygous" for a particular polymorphism is one in which the two copies of the gene contain different sequences at the polymorphic position.

A "polymorphism pattern" as used herein denotes a set of one or more polymorphisms, including without limitation single nucleotide polymorphisms, which may be contained in the sequence of a single gene or a plurality of genes. In the simplest case, a polymorphism pattern can consist of a single nucleotide polymorphism in only one position of one of two alleles of an individual . However, one has to look at both copies of a gene. A polymorphism pattern that is appropriate for assessing a particular aspect of cardiovascular status (*e.g.,* predisposition to hypertension) need not contain the same number (nor identity, of course) of polymorphisms as a polymorphism pattern that would be appropriate for assessing another aspect of cardiovascular status (*e.g.*, responsivity to ACE inhibitors for control of hypertension). A "test polymorphism pattern" as used herein is a polymorphism pattern determined for a human subject of undefined cardiovascular status. A "reference polymorphism pattern" as used herein is determined from a statistically significant correlation of patterns in a population of individuals with pre-determined cardiovascular status.

"Nucleic acid" or "polynucleotide" as used herein refers to purine- and pyrimidine-containing polymers of any length, either polyribonucleotides or polydeoxyribonucleotides or mixed polyribo-polydeoxyribo nucleotides. Nucleic acids include without limitation single- and double-stranded molecules, *i.e.*, DNA-DNA, DNA-

RNA and RNA-RNA hybrids, as well as "protein nucleic acids" (PNA) formed by conjugating bases to an amino acid backbone. This also includes nucleic acids containing modified bases and non-naturally occurring phosphoester analog bonds, such as phosphorothioates and thioesters. The term nucleic acid molecule, and in particular DNA or RNA molecule, refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear or circular DNA molecules (*e.g.*, restriction fragments), plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (*i.e.*, the strand having a sequence homologous to the mRNA). A "recombinant DNA molecule" is a DNA molecule that has undergone a molecular biological manipulation.

As used herein, the term "oligonucleotide" refers to a nucleic acid, generally of at least 10, preferably at least 15, and more preferably at least 20 nucleotides, that is hybridizable to a genomic DNA molecule, a cDNA molecule, or an mRNA molecule encoding a gene, cDNA, mRNA, or other nucleic acid of interest. Oligonucleotides can be labeled, *e.g.*, with ^{32}P-nucleotides or nucleotides to which a label, such as biotin, has been covalently conjugated. In one embodiment, a labeled oligonucleotide can be used as a probe to detect the presence of a nucleic acid. In another embodiment, oligonucleotides (one or both of which may be labeled) can be used as PCR primers, either for cloning full length or a fragment of a gene of interest, or to detect the presence of nucleic acids encoding the gene of interest. In a further embodiment, an oligonucleotide of the invention can form a triple helix with a double stranded sequence of interest in a DNA molecule. In still another embodiment, a library of oligonucleotides arranged on a solid support, such as a silicon wafer or chip, can be used to detect various polymorphisms of interest. Generally, oligonucleotides are prepared synthetically, preferably on a nucleic acid synthesizer. Accordingly, oligonucleotides can be prepared with non-naturally occurring phosphoester analog bonds, such as thioester bonds.

An "isolated" nucleic acid or polypeptide as used herein refers to a nucleic acid or polypeptide that is removed from its original environment (for example, its natural environment if it is naturally occurring). An isolated nucleic acid or polypeptide contains

less than about 50%, preferably less than about 75%, and most preferably less than about 90%, of the cellular components with which it was originally associated.

A nucleic acid or polypeptide sequence that is "derived from" a designated sequence refers to a sequence that corresponds to a region of the designated sequence. For nucleic acid sequences, this encompasses sequences that are identical to or complementary to the sequence.

A "probe" refers to a nucleic acid or oligonucleotide that forms a hybrid structure with a sequence in a target nucleic acid due to complementarity of at least one sequence in the probe with a sequence in the target nucleic acid. Generally, a probe is labeled so it can be detected after hybridization.

A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength (*see* Sambrook *et al.*, 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. For preliminary screening for homologous nucleic acids, low stringency hybridization conditions, corresponding to a T_m of 55°C, can be used, *e.g.*, 5x SSC, 0.1% SDS, 0.25% milk, and no formamide; or 30% formamide, 5x SSC, 0.5% SDS). Moderate stringency hybridization conditions correspond to a higher T_m, *e.g.*, 40% formamide, with 5x or 6x SCC. High stringency hybridization conditions correspond to the highest T_m, *e.g.*, 50% formamide, 5x or 6x SCC. Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater the value of T_m for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher T_m) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA. For hybrids of greater than 100 nucleotides in length, equations for calculating T_m have been derived (*see* Sambrook *et al.*, *supra*, 9.50-9.51). For hybridization with shorter nucleic acids, *i.e.*, oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity

(*see* Sambrook *et al.*, *supra*, 11.7-11.8). A minimum length for a hybridizable nucleic acid is at least about 10 nucleotides: preferably at least about 15 nucleotides; and more preferably the length is at least about 20 nucleotides.

In a specific embodiment, the term "standard hybridization conditions" refers to a T_m of 55°C, and utilizes conditions as set forth above. In a preferred embodiment, the T_m is 60°C; in a more preferred embodiment, the T_m is 65°C. In a specific embodiment, "high stringency" refers to hybridization and/or washing conditions at 68°C in 0.2XSSC, at 42°C in 50% formamide, 4XSSC, or under conditions that afford levels of hybridization equivalent to those observed under either of these two conditions.

A "gene" for a particular protein as used herein refers to a contiguous nucleic acid sequence corresponding to a sequence present in a genome which comprises (i) a "coding (or transcribed) region," which comprises exons (*i.e.*, sequences encoding a polypeptide sequence, or "protein-coding" or "transcribed sequences"), introns, sequences at the junction between exons and introns, and 5' and 3' untranslated regions (uTRs); and (ii) regulatory sequences, which flank the coding region at both 5' and 3' termini. For example, the "ACE gene" as used herein encompasses the regulatory and coding regions of the human gene encoding angiotensin converting enzyme. Similarly, the "AGT gene" encompasses regulatory and coding regions of the human gene encoding angiotensinogen and the "AT1 gene" encompasses regulatory and coding regions of the human gene encoding type I angiotensin II receptor. Typically, regulatory sequences according to the invention are located 5' (*i.e.*, upstream) of the coding region segment. The reference sequences, obtained from GenBank, which were used in practicing the present invention are shown in Table 1.

Table 1. GenBank Accession Numbers

Abbreviation	Compared master sequence	Numbering according to sequence entry in GenBank	SEQ ID NO.
AGT Regulatory Regions	X15323	X15323	122
AGT Coding Region	M24686 (exon 2) M24687 (exon 3) M24688 (exon 4) M24689 (exon 5) X62855 (intron 16)	Protein-coding sequences from exon 2-5 were spliced together as described in the GenBank entries. Nucleotide 1 is assigned to the first nucleotide of the initiator methionine codon. X62855	123 124 125 126 127
ACE Regulatory Region	X94359	X94359	128
ACE Coding Region	J04144	J04144 Nucleotide 1 is assigned to the first nucleotide of the initiator methionine codon.	129
AT1 Regulatory Region	U07144	U07144	130
AT1 Coding Region	S80239 (exon 3) S77410 (exon 5)	The protein-coding sequence of S80239 was spliced to position 288 of entry S77410. Nucleotide 1 is assigned to the first nucleotide of the initiator methionine codon in entry S80239.	131 132
Renin		M10030, X34914, X01391, X01734	133
Aldosterone synthase		D13752	134
AT2		U10273	135
β-adrenoceptor1 Regulatory Region (B1P)		X69168/g28421	136
β-adrenoceptor1 Coding Region (B1R)		J03019/g17899	137

β-adrenoceptor2 Regulatory Region (B2P)		M15169 J02728 M16106	138
β-adrenoceptor2 Coding Region (B2R)		Y00106/g29370	139
EndothelinA (ET_A) Receptor Coding		S57498	140

The present inventors have surprisingly and unexpectedly discovered the existence of genetic polymorphisms within the human gene encoding ACE, AT1, AGT, renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, or β-adrenoceptor which, singly or in combination, can be used to assess cardiovascular status, depending on which component of cardiovascular status is under evaluation. In accordance with the invention, the polymorphic pattern of the gene, alone or in combination with other genes encoding renin, ACE, AGT, AT1, AT2, aldosterone synthase, endothelin receptor, and β-adrenergic receptors 1 and 2 in an individual can predict the responsivity of the individual to particular therapeutic interventions and serve as an indicator of predisposition to various forms of cardiovascular disease. The invention provides methods for assessing cardiovascular status by detecting polymorphic patterns in an individual. The present invention also provides isolated nucleic acids derived from the gene which comprise these polymorphisms, including probes which hybridize specifically to polymorphic positions and primers that amplify the region of the gene in which the polymorphism is located; isolated polypeptides and peptides comprising polymorphic residues; and antibodies which specifically recognize ACE, AT1, AGT, renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, or β-adrenoceptor polypeptides containing one or more polymorphic amino acids.

Methods for Assessing Cardiovascular Status

The present invention provides diagnostic methods for assessing cardiovascular status in a human individual. The methods are carried out by comparing a polymorphic position or pattern ("test polymorphic pattern") within the individual's gene encoding ACE, AT1, AGT, renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, or β-adrenoceptor with the polymorphic patterns of humans

exhibiting a predetermined cardiovascular status ("reference polymorphic pattern"). If the cardiovascular status is the prediction of responsivity to a therapy, a single polymorphic position can provide a pattern for comparison. However, it is preferable to use more than one polymorphic position for the pattern to improve the accuracy of the prediction. If the cardiovascular status is predisposition to a cardiovascular syndrome, at least two, and preferably at least three, polymorphic positions are used to make the pattern. In addition, other polymorphisms in genes encoding angiotensin converting enzyme (ACE), angiotensinogen (AGT), type 1 angiotensin II receptor (AT1), type 2 angiotensin II receptor, renin, aldosterone synthase, endothelin, receptor or β-adrenergic receptors 1 and 2 can be used to establish a polymorphic pattern for the individual.

For any meaningful prediction, the polymorphic pattern of the individual is identical to the polymorphic pattern of individuals who exhibit particular status markers, cardiovascular syndromes, and/or particular patterns of response to therapeutic interventions.

In one embodiment, the method involves comparing an individual's test polymorphic pattern with reference polymorphic patterns of individuals who have been shown to respond positively or negatively to a particular therapeutic regimen. Therapeutic regimen as used herein refers to treatments aimed at the elimination or amelioration of symptoms and events associated cardiovascular disease. Such treatments include without limitation one or more of alteration in diet, lifestyle, and exercise regimen; invasive and noninvasive surgical techniques such as atherectomy, angioplasty, and coronary bypass surgery; and pharmaceutical interventions, such as administration of ACE inhibitors, angiotensin II receptor antagonists, diuretics, alpha-adrenoreceptor antagonists, cardiac glycosides, phosphodiesterase inhibitors, beta-adrenoreceptor antagonists, calcium channel blockers, HMG-CoA reductase inhibitors, imidazoline receptor blockers, endothelin receptor blockers, and organic nitrites. Interventions with pharmaceutical agents not yet known whose activity correlates with particular polymorphic patterns associated with cardiovascular disease are also encompassed. The present inventors have discovered that particular polymorphic patterns correlate with an individual's responsivity to ACE inhibitors (see, *e.g.*, Example 3 below). It is contemplated, for example, that patients who are candidates for a particular therapeutic regimen will be screened for polymorphic patterns that correlate with responsivity to that particular regimen.

In another embodiment, the method involves comparing an individual's polymorphic pattern with polymorphic patterns of individuals who exhibit or have exhibited one or more markers of cardiovascular disease, such as, *e.g.*, high blood pressure, abnormal electrocardiographic profile, myocardial infarction, unstable angina, stroke, or atherosclerosis (see, *e.g.*, Example 2 below) and drawing analogous conclusions as to the individual's responsivity to therapy, predisposition to developing a syndrome, etc., as detailed above.

Identification of Polymorphic Patterns

In practicing the methods of the invention, an individual's polymorphic pattern can be established *e.g.* by obtaining DNA from the individual and determining the sequence at a predetermined polymorphic position or positions in a gene, or more than one gene.

The DNA may be obtained from any cell source. Non-limiting examples of cell sources available in clinical practice include without limitation blood cells, buccal cells, cervicovaginal cells, epithelial cells from urine, fetal cells, or any cells present in tissue obtained by biopsy. Cells may also be obtained from body fluids, including without limitation blood, saliva, sweat, urine, cerebrospinal fluid, feces, and tissue exudates at the site of infection or inflammation. DNA is extracted from the cell source or body fluid using any of the numerous methods that are standard in the art. It will be understood that the particular method used to extract DNA will depend on the nature of the source.

Determination of the sequence of the extracted DNA at polymorphic positions is achieved by any means known in the art, including but not limited to direct sequencing, hybridization with allele-specific oligonucleotides, allele-specific PCR, ligase-PCR, HOT cleavage, denaturing gradient gel electrophoresis (DGGE), and single-stranded conformational polymorphism (SSCP). Direct sequencing may be accomplished by any method, including without limitation chemical sequencing, using the Maxam-Gilbert method; by enzymatic sequencing, using the Sanger method; mass spectrometry sequencing; and sequencing using a chip-based technology. See, *e.g.*, Little *et al.*, Genet. Anal., 1996, 6:151. Preferably, DNA from a subject is first subjected to amplification by polymerase chain reaction (PCR) using specific amplification primers.

In an alternate embodiment, biopsy tissue is obtained from a subject. Antibodies that are capable of distinguishing between different polymorphic forms of

ACE, AT1, AGT, renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, or β-adrenoceptor are then applied to samples of the tissue to determine the presence or absence of a polymorphic form specified by the antibody. The antibodies may be polyclonal or monoclonal, preferably monoclonal. Measurement of specific antibody binding to cells may be accomplished by any known method, *e.g.*, quantitative flow cytometry, or enzyme-linked or fluorescence-linked immunoassay. The presence or absence of a particular polymorphism or polymorphic pattern, and its allelic distribution (*i.e.*, homozygosity vs. heterozygosity) is determined by comparing the values obtained from a patient with norms established from populations of patients having known polymorphic patterns.

In another alternate embodiment, RNA is isolated from biopsy tissue using standard methods well known to those of ordinary skill in the art such as guanidium thiocyanate-phenol-chloroform extraction (Chomocyznski *et al.*, Anal. Biochem., 1987, 162:156). The isolated RNA is then subjected to coupled reverse transcription and amplification by polymerase chain reaction (RT-PCR), using specific oligonucleotide primers that are specific for a selected polymorphism. Conditions for primer annealing are chosen to ensure specific reverse transcription and amplification; thus, the appearance of an amplification product is diagnostic of the presence of a particular polymorphism. In another embodiment, RNA is reverse-transcribed and amplified, after which the amplified sequences are identified by, *e.g.*, direct sequencing. In still another embodiment, cDNA obtained from the RNA can be cloned and sequenced to identify a polymorphism.

Establishing Reference Polymorphism Patterns

In practicing the present invention, the distribution of polymorphic patterns in a large number of individuals exhibiting particular cardiovascular status is determined by any of the methods described above, and compared with the distribution of polymorphic patterns in patients that have been matched for age, ethnic origin, and/or any other statistically or medically relevant parameters, who exhibit quantitatively or qualitatively different cardiovascular status. Correlations are achieved using any method known in the art, including nominal logistic regression or standard least squares regression analysis. In this manner, it is possible to establish statistically significant correlations between particular polymorphic patterns and particular cardiovascular statuses. It is further possible to establish statistically significant correlations between particular

polymorphic patterns and changes in cardiovascular status such as, would result, *e.g.*, from particular treatment regimens. Thus, it is possible to correlate polymorphic patterns with responsivity to particular treatments.

A statistically significant correlation preferably has a "p" value of less than or equal to 0.05. Any standard statistical method can be used to calculate these values, such as the normal Student's T Test, or Fischer's Exact Test.

The identity and number of polymorphisms to be included in a reference pattern depends not only on the prevalence of a polymorphism and its predictive value for the particular use, but also on the value of the use and its requirement for accuracy of prediction. The greater the predictive value of a polymorphism, the lower the need for inclusion of more than one polymorphism in the reference pattern. However, if a polymorphism is very rare, then its absence from an individual's pattern might provide no indication as to whether the individual has a particular status. Under these circumstances, it might be advisable to select instead two or more polymorphisms which are more prevalent. Even if none of them has a high predictive value on its own, the presence of both (or all three) of them might be sufficiently predictive for the particular purpose.

If for example the use for a reference pattern is prediction of response to a drug, and among the afflicted population only a 30% response to the drug is observed, the reference pattern need only permit selection of a population that improves the response rate by 10% to provide a significant improvement in the state of the art. On the other hand, if the use for the reference pattern is selection of subjects for a particular clinical study, the pattern should be as selective as possible and should therefore include a plurality of polymorphisms that together provide a high predictive accuracy for the intended response.

In establishing reference polymorphism patterns, it is desirable to use a defined population. For example, tissue libraries collected and maintained by state or national departments of health can provide a valuable resource, since genotypes determined from these samples can be matched with medical history, and particularly cardiovascular status, of the individual. Such tissue libraries are found, for example, in Sweden, Iceland, Norway, and Finland. As can be readily understood by one of ordinary skill in the art, specific polymorphisms may be associated with a closely linked population. However, other polymorphisms in the same gene may correlate with cardiovascular status of other genetically related populations. Thus, in addition to the

specific polymorphisms provided in the instant application, the invention identifies genes in which any polymorphisms can be used to establish reference and test polymorphism patterns for evaluating cardiovascular status of individuals in the population.

In a specific embodiment, DNA samples can be obtained form a well defined population, such as 277 Caucasian males born in Uppsala, Sweden between 1920 and 1924. In a specific embodiment, such individuals are selected for the test population based on their medical history, *i.e.*, they were either (i) healthy, with no signs of cardiovascular disease (100); or (ii) had suffered one of acute myocardial infarction (68), silent myocardial infarction (34), stroke (18), stroke and acute myocardial infarction (19), or high blood pressure at age 50 (39). DNA samples are obtained from each individual.

In a specific embodiment, DNA sequence analysis can be carried out by: (i) amplifying short fragments of each of the genes using polymerase chain reaction (PCR) and (ii) sequencing the amplified fragments. The sequences obtained from each individual can then be compared with the first known sequences, *e.g.*, as set forth in Table 1, to identify polymorphic positions.

Comparing Test Patterns to Reference Patterns

As noted above, the test pattern from an individual can be compared to a reference pattern established for a predetermined cardiovascular status. Identity between the test pattern and the reference pattern means that the tested individual has a probability of having the same cardiovascular status as that represented by the reference pattern. As discussed above, this probability depends on the prevalence of the polymorphism and the statistical significance of its correlation with a cardiovascular status.

Polymorphic Positions

Polymorphic positions in the genes encoding ACE, AT1, AGT, renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, or β-adrenoceptor which are encompassed by the invention can be identified by determining the DNA sequence of all or part of the gene in a multiplicity of individuals in a population. DNA sequence determination may be achieved using any conventional method, including, *e.g.*, chemical or enzymatic sequencing.

The polymorphic positions of genes for use in the invention include without limitation those listed below, whose numbering corresponds to the GenBank sequences listed in Table 1.

(i) ACE: positions in the regulatory region (designated ACR) numbered 5106, 5349, and 5496; positions in the coding region (designated ACE) numbered 375, 582, 731, 1060. 1215, 2193, 2328, 2741, 3132, 3387, 3503, and 3906; and position 1451 as numbered in GenBank entry X62855.

(ii) AGT: positions in the regulatory region (designated AGR) numbered 395, 412, 432, 449, 692, 839, 1007, 1072, 1204, and 1218; positions in the coding region (designated AGT) numbered 273, 620, 803, 912, 997, 1116, and 1174; and position 49 as numbered in GenBank entry M24688.

(iii) AT1: positions in the regulatory region (designated ATR) numbered 1427, 1756, 1853, 2046, 2354, 2355, and 2415; and positions in the coding region (designated AT1) numbered 449, 678, 1167, and 1271.

(iv) Renin: A mutant renin gene in familial elevation of prorenin, a point mutation in the last exon of the gene (exon 10), has been identified (Villard *et al.*, J. Biol. Chem., 1994, 269:30307-12). A cytosine to thymine transition creates a premature stop codon at position 387 resulting in a truncated form of renin with 20 amino acids deleted from the carboxyl terminus.

(v) Aldosterone synthase: A position in the promoter region of aldosterone synthase, position -344 (with the initiation codon starting at 1) has been reported by Cambien *et al.* at the International Meeting on Hypertension held in Amsterdam in June 1998.

β-adrenergic receptor-1, positions in the regulatory region (designated BP1) numbered 2238, 2440, 2493, 2502, 2577, 2585, 2693, 2724, and 2757; and positions in the coding region (designated BR1) numbered 231, 758, 1037, 1251, 1403, and 1528.

β-adrenergic receptor-2, positions in the regulatory region (designated B2P) numbered 932, 934, 987, 1006, 1120, 1221, 1541, and 1568; and positions in the coding region (designated B2R) numbered 839, 872, 1045, 1284, 1316, 1846, 1891, 2032, 2068, and 2070.

Endothelin receptor type A coding region (designated ET_A) numbered 969, 1005, 1146, and 2485.

In preferred embodiments, the base at each of the above polymorphic positions is one of:

(i) ACE Regulatory Region: 5106C, 5106T, 5349A, 5349T, 5496T, and 5496C;

(ii) ACE Coding Region: 375A, 375C, 582C, 582T, 731A, 731G, 1060G, 1060A, 1215C, 1215T, 2193G, 2193A, 2328A, 2328G, 2741G, 2741T, 3132C, 3132T, 3387T, 3387C, 3503G, 3503C, 3906G, and 3906A; and a deletion of nucleotides 1451-1783 as numbered in GenBank entry X62855;

(iii) AGT Regulatory Region: 395T, 395A, 412C, 412T, 432G, 432A, 449T, 449C, 692C, 692T, 839G, 839A, 1007G, 1007A, 1072G, 1072A, 1204C, 1204A, 1218A, 1218G;

(iv) AGT Coding Region: 273C, 273T, 620C, 620T, 803T, 803C, 912C, 912T, 997G, 997C, 1116G, 1116A, 1174C, and 1174A; and A or G at position 49 in GenBank entry M24688;

(v) AT1 Regulatory Region: 1427A, 1427T, 1756T, 1756A, 1853T, 1853G, 2046T, 2046C, 2354A, 2354C, 2355G, 2355C, 2415A and 2415G; and

(vi) AT1 Coding Region: 449G, 449C, 678T, 678C, 1167A, 1167G, 1271A, and 1271C.

(vii) β-adrenergic receptor-1 regulatory region: 2238 G, 2238 A, 2577 C, 2257 T, 2757 A, and 2757 G.

(viii) β-adrenergic receptor-1 coding region: 231 A, 231 G, 758 C, 758 T, 1251 C, 1251 G, 1403 A, 1403 G, 1528 C, and 1528 A.

(ix) β-adrenergic receptor-2 regulatory region: 934 A, 934 G, 987 C, 987 G, 1006 A, 1006 G, 1120 C, 1120 G, 1221 C, 1221 T, 1541 C, 1541 T, 1568 C, and 1568 T.

(x) β-adrenergic receptor-2 coding region: 839 A, 839 G, 872 C, 872 G, 1045A, 1045 G, 1284 C, 1284 T, 1316 A, 1316 C, 1846 C, 1846 G, 2032 A, 2032 G, 2068 no insert, 2068 G, 2068 C, 2070 no insert, and 2070 C.

(xi) Endothelin receptor type A: 969 C, 969 T, 1005 A, 1005 G, 1146 A, 1146 G, 2485 T, and 2485 C.

An individual may be homozygous or heterozygous for a particular polymorphic position (see, *e.g.*, Table 6 below).

Non-limiting examples of polymorphic patterns comprising one or more polymorphism in ACE, AGT, and/or AT1 genes according to the invention include the following, which were correlated with an increased incidence of clinical signs of cardiovascular disease:

ACR 5349 A/T, AGR 1218 A; ACR 5496 C, AGR 1204 A/C; ACR 5496 C/T, AGR 1218 A, AGT 620 C/T; ACE 2193 A, AGR 1204 C, ACE 2328 G; ACE 2193 A, AGR 1204 A/C; ACE 3387 T, AGR 1218 A; ACE 3387 T, AGT 620 C/T; AGR 1204 A/C, AT1 678 C/T; AGR 1204 A/C, AT1 1271 A/C; ACE 1215 C, AGR 1204 A/C; AGR 1204 A/C, AT1 1167 A, ACE 3906 A/G; AGR 1204 A, AGT 620 C, AT1 1271 A, AT1 1167 A, AGR 395 A/T; AGR 1204 A/C, AGT 620 C/T, AT1 1271 A/C, AT1 1167 A, AGR 395 T; AGR 1204 A/C, AGT 620 C/T, AT1 1271 A/C, AT1 1167 A/G, AGR 395 T; AGR 1204 A, AT1 678 C, AT1 1167 A, AGR 395 A/T; AGR 1204 A/C, AT1 678 C/T, AT1 1167 A, AGR 395 T; AGT 620 C/T, AT1 1271 A/C, AT1 1167 A, AGR 395 T; AGT 620 C/T, AT1 1271 A/C, AT1 1167 A/G, AGR 395 T; AGT 620 C, AT1 1271 A, AT1 1167 A, AGR 395 A/T; AGT 620 C, AT1 678 A, AT1 1167 A, AGR 395 A/T; AGT 620 C/T, AT1 678 C/T; AT1 1167 A, AGR 395 T; ACE 2193 A, AGR 1218 A, AGT 803 A; ACE 2193 A, AGT 620 C/T; ACE 2328 G, AGT 620 C/T; ACE 3387 T, AGR 1204 A/C; ACE 2193 A, ACE 2328 G, AGR 1204 C; ACE 2193 A/G, AGR 1072 G/G, AT1 1167 A/A. Additional polymorphism patterns are shown in the Tables in Examples 4 and 5, below.

Polymorphism Patterns Correlated With ACE Inhibitor Responsiveness

The following table lists a set of polymorphism patterns that have been found to correlate with responsiveness to ACE inhibitor treatment:

Response to ACE-Inhibitor Treatment

Position 1	Genotype 1	Position 2	Genotype 2	Position 3	Genotype 3
ACE:2193:	A/G	AGR:1072:	G/G	AT1:1167:	A/A
ACE:2193:	A/G	AGR:1072:	G/G	ACE:1060:	G/G
ATR:2354:	A/A	AT1:678:	C/T	AT1:1167:	A/A
ACR:5496:	C/T	AGR:1204:	A/A	AGR:839:	G/G
ACR:5496:	C/T	AGR:1204:	A/A	AGT:620:	C/C
ACR:5496:	C/T	ACE:1060:	G/G	AGR:449:	C/T

Position 1	Genotype 1	Position 2	Genotype 2	Position 3	Genotype 3
ACR:5496:	C/T	ACE:1215:	C/T	AGR:1204:	A/A
ACR:5496:	C/T	ACE:3906:	A/G	AGR:1218:	A/G
ACR:5496:	C/T	AGR:449:	C/T	AGR:839:	G/G
ACR:5496:	C/T	AGR:449:	C/T	AT1:1271:	A/A
ACR:5496:	C/T	AGR:449:	C/T	AGR:1072:	G/G
ACR:5496:	C/T	AGR:1072:	G/G	AGR:1204:	A/A
ACE:1060:	G/G	AGR:449:	C/T	AGT:620:	C/C
ACE:1060:	G/G	AGR:1007:	G/G	AT1:678:	C/T
ACE:1215:	C/T	ACE:3906:	A/G	AGR:1218:	A/G
AGR:449:	C/T	AGR:1204:	A/A	AGT:620:	C/C
AGR:449:	C/T	AGR:1204:	A/A	AGT:1116:	G/G
AGR:449:	C/T	AGR:839:	G/G	AGT:620:	C/C
AGR:449:	C/T	AGT:620:	C/C	AGT:1116:	G/G
AGR:449:	C/T	AGT:1116:	G/G	AT1:1271:	A/A
AGR:449:	C/T	AGR:1072:	G/G	AGT:620:	C/C
AGR:1007:	G/C	AGR:1072:	G/G	AT1:678:	C/T
AGR:1072:	G/G	AGR:1204:	A/A	AGT:1116:	G/G
ACR:5496:	C/T	AGR:1204:	A/A	AT1:1167:	A/A
AGR:1007:	G/G	AGR:1204:	A/A	AGT:1116:	G/G

Polymorphism Patterns of Correlated With ACE Inhibitor Non-responsiveness

The following table lists a set of polymorphism patterns that have been found to correlate with non-responsiveness to ACE inhibitor treatment:

Non-Response to ACE-Inhibitor Treatment

Position 1	Genotype 1	Position 2	Genotype 2	Position 3	Genotype 3
ACE:1060:	G/G	AGR:1204:	A/C	AGT:620:	C/C
ACE:1215:	C/T	ACE:2193:	A/G	AGR:1204 :	A/C
ACE:2193:	A/G	ATR:2046:	C/T	AT1:678:	C/C

Position 1	Genotype 1	Position 2	Genotype 2	Position 3	Genotype 3
ACE:2193:	A/G	AT1:678:	C/C	AT1:1271:	A/C

Polymorphism Patterns Correlated With Predisposition to MI

The following table lists a set of polymorphism patterns that have been found to correlate with predisposition to myocardial infarction:

Predisposition to MI

Position 1	Genotype 1	Position 2	Genotype 2	Position 3	Genotype 3
ACE:2193:	A/A	AGR:1204:	A/C	-	-
AGR:449:	T/T	AGR:1204:	A/C	AT1:1271:	A/C
ACE:2193:	A/G	AGR:620:	C/T	AGR:1116:	G/G
ACE:2193:	A/G	AGR:449:	T/T	AT1:1271:	A/C
ACR:5349:	A/A	AGR:449:	T/T	AT1:1271:	A/C
ACE:2193:	A/A	AGR:620:	C/T	AT1:1271:	A/A

Polymorphism Patterns Correlated With Predisposition to Stroke

The following table lists a set of polymorphism patterns that have been found to correlate with predisposition to stroke:

Predisposition to Stroke

Position 1	Genotype 1	Position 2	Genotype 2	Position 3	Genotype 3
ACE:2193:	A/A	AGR:395:	A/T	-	-
AGR:1007:	A/G	AGR:1072:	G/G	AT1:1167:	A/A
AGR:395:	A/T	AGR:1072:	G/G	AGR:1218:	A/G

Polymorphism Patterns in β-Adrenergic Receptor Genes

The following table lists a set of polymorphism patterns that have been found in β-adrenergic receptor genes:

Positions carrying genetic variation in the Beta adrenergic receptors 1 and 2.

B1P	B1R	B2R	B2P
2238 C/A	231 A/G	839 A/G	932
2440	758 C/T 1037	872 C/G	934 A/G
2493	1251 C/G	1045 A/G	987 C/G 1006 A/G
2502	1403 A/G	1284 C/T	1120 C/G
2577 C/T	1528 C/A	1316 A/C	1221 C/T
2585		1846 C/G	1541 C/T
2693		1891	1568 C/T
2724		2032 A/G	
2757 A/G		2068 no insert, G/G, C/G 2070 no insert /C	

B1P: Beta adrenergic receptor 1, regulatory promoter region.

B1R: Beta adrenergic receptor 1, coding region.

B2P: Beta adrenergic receptor 2, regulatory promoter region.

B2R: Beta adrenergic receptor 2, coding region.

A number of different polymorphisms have been identified in the type 2 β-adrenoceptor. All of these differed from the wild type sequence by a single base change. Four of the polymorphisms alter the amino acid sequence of the receptor protein (Hall, Thorax, 1996, 51:351-353). The amino acid sequence modifications are described in greater detail below:

Arg16-Gly: The Gly16 variant undergoes an enhanced agonist-promoted down regulation as compared to wild type but the coupling to adenylyl cyclase and agonist binding are maintained (Liggett, Am. J. Respir. Crit. Care Med., 1997, 156:S 156-S162).

Gln27-Glu: The Glu27 variant displays very little agonist-promoted downregulation and the coupling to adenylyl cyclase and agonist binding are maintained (*id.*).

Val34-Met: Met34 is very rare. No altering of receptor function has been found (*id.*).

Thr164-Ile: Uncommon (about 5%). The Ile164 variant shows depressed coupling to adenylyl cyclase and decreased affinities for agonists with hydroxyl groups on their β-carbons, such as epinephrine, norepinephrine, and isoproterenol compared to wild type (*id.*).

The polymorphism at nucleic acid 523 (CGG-AGG) might be linked with one of the other functional polymorphisms (*id.*).

There are no differences in frequency of these polymorphisms between the normal group and those with asthma but they have been correlated to differences in response to treatment with agonists in asthma, *e.g.*, the Gly16 variant undergoes an enhanced agonist-promoted downregulation compared to wild type (*id.*).

Polymorphism Patterns in Endothelin Receptor Type A Gene

The following table lists a set of polymorphism patterns that have been found in the coding region of the endothelin receptor type A gene:

Position 969	Position 1005	Position 1146	Position 2485
C/C	A/A	A/A	T/T
C/T	A/G	A/G	T/C
T/T	G/G		

Isolated Polymorphic Nucleic Acids, Vectors, Probes & Primers and Arrays

Vectors for Expression of Polymorphic Variants

The present invention provides isolated nucleic acids comprising the polymorphic positions described above for the human genes encoding ACE, AT1, AGT, renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, and β-adrenoceptor; vectors comprising the nucleic acids; and transformed host cells comprising the vectors. The invention also provides probes which are useful for detecting these polymorphisms.

The nucleic acids encoding a gene comprising a polymorphism that is useful for determining cardiovascular status of an individual is particularly valuable for screening, whether by direct screening of the nucleic acid with the polymorphism, or by screening the polypeptide expressed by that nucleic acid.

In practicing the present invention, many conventional techniques in molecular biology, microbiology, and recombinant DNA, are used. Such techniques are well known and are explained fully in, for example, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Second Edition, 1989 (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); *DNA Cloning: A Practical Approach*, Volumes I and II, 1985 (D.N. Glover ed.); *Oligonucleotide Synthesis,* 1984, (M.L. Gait ed.); *Nucleic Acid Hybridization*, 1985, (Hames and Higgins); Ausubel *et al.*, *Current Protocols in Molecular Biology*, 1997 (John Wiley and Sons); and *Methods in Enzymology* Vol. 154 and Vol. 155 (Wu and Grossman, and Wu, eds., respectively).

Insertion of nucleic acids (typically DNAs) comprising the sequences of the present invention into a vector is easily accomplished when the termini of both the DNAs and the vector comprise compatible restriction sites. If this cannot be done, it may be necessary to modify the termini of the DNAs and/or vector by digesting back single-stranded DNA overhangs generated by restriction endonuclease cleavage to produce blunt ends, or to achieve the same result by filling in the single-stranded termini with an appropriate DNA polymerase.

Alternatively, any site desired may be produced, *e.g.*, by ligating nucleotide sequences (linkers) onto the termini. Such linkers may comprise specific oligonucleotide sequences that define desired restriction sites. Restriction sites can also be generated by the use of the polymerase chain reaction (PCR). *See, e.g.*, Saiki *et al.*, Science, 1988, 239:48. The cleaved vector and the DNA fragments may also be modified if required by homopolymeric tailing.

The nucleic acids may be isolated directly from cells or may be chemically synthesized using known methods. Alternatively, the polymerase chain reaction (PCR) method can be used to produce the nucleic acids of the invention, using either chemically synthesized strands or genomic material as templates. Primers used for PCR can be synthesized using the sequence information provided herein and can further be designed to introduce appropriate new restriction sites, if desirable, to facilitate incorporation into a given vector for recombinant expression.

The nucleic acids of the present invention may be flanked by native gene sequences, or may be associated with heterologous sequences, including promoters, enhancers, response elements, signal sequences, polyadenylation sequences, introns, 5'- and 3'- noncoding regions, and the like.

The invention also provides nucleic acid vectors comprising the disclosed genes or derivatives or fragments thereof. A large number of vectors, including plasmid and fungal vectors, have been described for replication and/or expression in a variety of eukaryotic and prokaryotic hosts, and may be used for gene therapy as well as for simple cloning or protein expression. Non-limiting examples of suitable vectors include without limitation pUC plasmids, pET plasmids (Novagen, Inc., Madison, WI), or pRSET or pREP (Invitrogen, San Diego, CA), and many appropriate host cells, using methods disclosed or cited herein or otherwise known to those skilled in the relevant art. The particular choice of vector/host is not critical to the practice of the invention.

Suitable host cells may be transformed/transfected/infected as appropriate by any suitable method including electroporation, $CaCl_2$ mediated DNA uptake, calcium phosphate precipitation, fungal or viral infection, lipofection, microinjection, microprojectile, or other established methods. Appropriate host cells included bacteria, archebacteria, fungi, especially yeast, and plant and animal cells, especially mammalian cells. A large number of transcription initiation and termination regulatory regions have been isolated and shown to be effective in the transcription and translation of heterologous proteins in the various hosts. Examples of these regions, methods of isolation, manner of manipulation, *etc.* are known in the art. Under appropriate expression conditions, host cells can be used as a source of recombinantly produced ACE-, AGT-, or AT1-derived peptides and polypeptides.

Nucleic acids encoding ACE-, AGT-, or AT1-derived gene sequences may also be introduced into cells by recombination events. For example, such a sequence can be introduced into a cell and thereby effect homologous recombination at the site of an endogenous gene or a sequence with substantial identity to the gene. Other recombination-based methods such as nonhomologous recombinations or deletion of endogenous genes by homologous recombination may also be used.

Oligonucleotides

The nucleic acids of the present invention find use as probes for the detection of genetic polymorphisms, as primers for the expression of polymorphisms, or in molecular library arrays for high throughput screening.

Probes in accordance with the present invention comprise without limitation isolated nucleic acids of about 10 - 100 bp, preferably 15-75 bp and most

preferably 17-25 bp in length, which hybridize at high stringency to one or more of the gene-derived polymorphic sequences disclosed herein or to a sequence immediately adjacent to a polymorphic position. Furthermore, in some embodiments a full-length gene sequence may be used as a probe. In one series of embodiments, the probes span the polymorphic positions in the genes disclosed above. In another series of embodiments, the probes correspond to sequences immediately adjacent to the polymorphic positions.

The oligonucleotide nucleic acids may also be modified by many means known in the art. Non-limiting examples of such modifications include methylation, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (*e.g.*, methyl phosphonates, phosphotriesters, phosphoroamidates, carbamates, *etc.*) and with charged linkages (*e.g.*, phosphorothioates, phosphorodithioates, *etc.*). Nucleic acids may contain one or more additional covalently linked moieties, such as, for example, proteins (*e.g.*, nucleases, toxins, antibodies, signal peptides, poly-L-lysine, *etc.*), intercalators (*e.g.*, acridine, psoralen, *etc.*), chelators (*e.g.*, metals, radioactive metals, iron, oxidative metals, *etc.*), and alkylators. PNAs are also included. The nucleic acid may be derivatized by formation of a methyl or ethyl phosphotriester or an alkyl phosphoramidate linkage. Furthermore, the nucleic acid sequences of the present invention may also be modified with a label capable of providing a detectable signal, either directly or indirectly. Exemplary labels include radioisotopes, fluorescent molecules, biotin, and the like.

PCR amplification of gene segments that contain a polymorphism provides a powerful tool for detecting the polymorphism. The oligonucleotides of the invention can also be used as PCR primers to amplify gene segments containing a polymorphism of interest. The amplified segment can be evaluated for the presence or absence of a polymorphism by restriction endonuclease activity, SSCP, or by direct sequencing. In another embodiment, the primer is specific for a polymorphic sequence on the gene. If the polymorphism is present, the primer can hybridize and DNA will be produced by PCR. However, if the polymorphism is absent, the primer will not hybridize, and no DNA will be produced. Thus, PCR can be used to directly evaluate whether a polymorphism is present or absent.

Molecular library arrays of oligonucleotides (including oligonucleotides with modifications as described above) are another powerful tool for rapidly assessing whether one or more polymorphisms are present in a *ACE, AT1, AGT, renin, aldosterone*

synthase, type-2 angiotensin II receptor, endothelin receptor, or *β-adrenoceptor* gene, preferably in combination with other genes. Molecular library arrays are disclosed in US Patents No. 5,677,195, No. 5,599,695, No. 5,545,531, and No. 5,510,270.

Polymorphic Polypeptides and Polymorphism-Specific Antibodies

The present invention encompasses isolated peptides and polypeptides encoded by all or a portion of a gene encoding a polypeptide selected from the group consisting of ACE, AT1, AGT, renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, and β-adrenoceptor, comprising polymorphic positions disclosed above. In one preferred embodiment, the peptides and polypeptides are useful screening targets to identify cardiovascular drugs. In another preferred embodiment, the peptides and polypeptides are capable of eliciting antibodies in a suitable host animal that react specifically with a polypeptide comprising the polymorphic position and distinguish it from other polypeptides having a different amino acid sequence at that position.

Polypeptides according to the invention are preferably at least five or more residues in length, preferably at least fifteen residues. Methods for obtaining these polypeptides are described below. Many conventional techniques in protein biochemistry and immunology are used. Such techniques are well known and are explained in *Immunochemical Methods in Cell and Molecular Biology*, 1987 (Mayer and Waler, eds; Academic Press, London); Scopes, *Protein Purification: Principles and Practice*, Second Edition 1987 (Springer-Verlag, N.Y.) and *Handbook of Experimental Immunology*, Volumes I-IV 1986 (Weir and Blackwell eds.).

Nucleic acids comprising protein-coding sequences can be used to direct the recombinant expression of ACE, AT1, AGT, renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, or β-adrenoceptor-derived polypeptides in intact cells or in cell-free translation systems. The known genetic code, tailored if desired for more efficient expression in a given host organism, can be used to synthesize oligonucleotides encoding the desired amino acid sequences. The polypeptides may be isolated from human cells, or from heterologous organisms or cells (including, but not limited to, bacteria, fungi, insect, plant, and mammalian cells) into which an appropriate protein-coding sequence has been introduced and expressed. Furthermore, the polypeptides may be part of recombinant fusion proteins.

Peptides and polypeptides may be chemically synthesized by commercially available automated procedures. including, without limitation, exclusive solid phase synthesis, partial solid phase methods, fragment condensation or classical solution synthesis. The polypeptides are preferably prepared by solid phase peptide synthesis as described by Merrifield, J. Am. Chem. Soc., 1963, 85:2149.

Methods for polypeptide purification are well-known in the art, including, without limitation, preparative disc-gel electrophoresis, isoelectric focusing, HPLC, reversed-phase HPLC, gel filtration, ion exchange and partition chromatography, and countercurrent distribution. For some purposes, it is preferable to produce the polypeptide in a recombinant system in which the protein contains an additional sequence tag that facilitates purification, such as, but not limited to, a polyhistidine sequence. The polypeptide can then be purified from a crude lysate of the host cell by chromatography on an appropriate solid-phase matrix. Alternatively, antibodies produced against ACE, AT1, AGT, renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, or β-adrenoceptor or against peptides derived therefrom, can be used as purification reagents. Other purification methods are possible.

The present invention also encompasses derivatives and homologues of the polypeptides. For some purposes, nucleic acid sequences encoding the peptides may be altered by substitutions, additions, or deletions that provide for functionally equivalent molecules, *i.e.*, function-conservative variants. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of similar properties, such as, for example, positively charged amino acids (arginine, lysine, and histidine); negatively charged amino acids (aspartate and glutamate); polar neutral amino acids; and non-polar amino acids.

The isolated polypeptides may be modified by, for example, phosphorylation, sulfation, acylation, or other protein modifications. They may also be modified with a label capable of providing a detectable signal, either directly or indirectly, including, but not limited to, radioisotopes and fluorescent compounds.

The present invention also encompasses antibodies that specifically recognize the polymorphic positions of the invention and distinguish a peptide or polypeptide containing a particular polymorphism from one that contains a different sequence at that position. Such polymorphic position-specific antibodies according to the present invention include polyclonal and monoclonal antibodies. The antibodies may be

elicited in an animal host by immunization with ACE, AT1, AGT, renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, or β-adrenoceptor-derived immunogenic components or may be formed by *in vitro* immunization of immune cells. The immunogenic components used to elicit the antibodies may be isolated from human cells or produced in recombinant systems. The antibodies may also be produced in recombinant systems programmed with appropriate antibody-encoding DNA. Alternatively, the antibodies may be constructed by biochemical reconstitution of purified heavy and light chains. The antibodies include hybrid antibodies (*i.e.*, containing two sets of heavy chain/light chain combinations, each of which recognizes a different antigen), chimeric antibodies (*i.e.*, in which either the heavy chains, light chains, or both, are fusion proteins), and univalent antibodies (*i.e.*, comprised of a heavy chain/light chain complex bound to the constant region of a second heavy chain). Also included are Fab fragments, including Fab' and $F(ab)_2$ fragments of antibodies. Methods for the production of all of the above types of antibodies and derivatives are well-known in the art and are discussed in more detail below. For example, techniques for producing and processing polyclonal antisera are disclosed in Mayer and Walker, *Immunochemical Methods in Cell and Molecular Biology*, 1987 (Academic Press, London). The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. See, *e.g.*, Schreier *et al.*, *Hybridoma Techniques*, 1980; U.S. Patent Nos. 4,341,761; 4,399,121; 4,427,783; 4,444,887; 4,466,917; 4,472,500; 4,491,632; and 4,493,890. Panels of monoclonal antibodies produced against ACE, AGT, or AT1-derived epitopes can be screened for various properties; *i.e.* for isotype, epitope affinity, *etc.*

The antibodies of this invention can be purified by standard methods, including but not limited to preparative disc-gel electrophoresis, isoelectric focusing, HPLC, reversed-phase HPLC, gel filtration, ion exchange and partition chromatography, and countercurrent distribution. Purification methods for antibodies are disclosed, *e.g.*, in *The Art of Antibody Purification*, 1989 (Amicon Division, W.R. Grace & Co.) General protein purification methods are described in *Protein Purification: Principles and Practice*, R.K. Scopes, Ed., 1987 (Springer-Verlag, New York, NY).

Methods for determining the immunogenic capability of the disclosed sequences and the characteristics of the resulting sequence-specific antibodies and immune

cells are well-known in the art. For example, antibodies elicited in response to a peptide comprising a particular polymorphic sequence can be tested for their ability to specifically recognize that polymorphic sequence, *i.e.*, to bind differentially to a peptide or polypeptide comprising the polymorphic sequence and thus distinguish it from a similar peptide or polypeptide containing a different sequence at the same position.

Diagnostic Methods and Kits

The present invention provides kits for the determination of the sequence at a polymorphic position or positions within the *ACE, AT1, AGT, renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor,* or *β-adrenoceptor* gene in an individual, in combination with determination of the sequence at polymorphism positions of other genes. The kits comprise a means for determining the sequence at the polymorphic positions, and may optionally include data for analysis of polymorphic patterns. The means for sequence determination may comprise suitable nucleic acid-based and immunological reagents (see below). Preferably, the kits also comprise suitable buffers, control reagents where appropriate, and directions for determining the sequence at a polymorphic position. The kits may also comprise data for correlation of particular polymorphic patterns with desirable treatment regimens or other indicators.

Nucleic Acid-Based Diagnostic Methods and Kits

The invention provides nucleic acid-based methods for detecting polymorphic patterns in a biological sample. The sequence at particular polymorphic positions in the genes is determined using any suitable means known in the art, including without limitation hybridization with polymorphism-specific probes and direct sequencing.

The present invention also provides kits suitable for nucleic acid-based diagnostic applications. In one embodiment, diagnostic kits include the following components:

(i) *Probe DNA:* The probe DNA may be pre-labelled; alternatively, the probe DNA may be unlabelled and the ingredients for labelling may be included in the kit in separate containers; and

(ii) *Hybridization reagents:* The kit may also contain other suitably packaged reagents and materials needed for the particular hybridization protocol, including solid-phase matrices, if applicable, and standards.

In another embodiment, diagnostic kits include:

(i) *Sequence determination primers:* Sequencing primers may be pre-labelled or may contain an affinity purification or attachment moiety; and

(ii) *Sequence determination reagents:* The kit may also contain other suitably packaged reagents and materials needed for the particular sequencing protocol. In one preferred embodiment, the kit comprises a panel of sequencing primers, whose sequences correspond to sequences adjacent to the polymorphic positions.

Antibody-Based Diagnostic Methods and Kits

The invention also provides antibody-based methods for detecting polymorphic patterns in a biological sample. The methods comprise the steps of: (i) contacting a sample with one or more antibody preparations, wherein each of the antibody preparations is specific for a particular polymorphic form of the gene under conditions in which a stable antigen-antibody complex can form between the antibody and antigenic components in the sample; and (ii) detecting any antigen-antibody complex formed in step (i) using any suitable means known in the art, wherein the detection of a complex indicates the presence of the particular polymorphic form in the sample.

Typically, immunoassays use either a labelled antibody or a labelled antigenic component (*e.g.*, that competes with the antigen in the sample for binding to the antibody). Suitable labels include without limitation enzyme-based, fluorescent, chemiluminescent, radioactive, or dye molecules. Assays that amplify the signals from the probe are also known, such as, for example, those that utilize biotin and avidin, and enzyme-labelled immunoassays, such as ELISA assays.

The present invention also provides kits suitable for antibody-based diagnostic applications. Diagnostic kits typically include one or more of the following components:

(i) *Polymorphism-specific antibodies:* The antibodies may be pre-labelled; alternatively, the antibody may be unlabelled and the ingredients for labelling may be included in the kit in separate containers, or a secondary, labelled antibody is provided; and

(ii) *Reaction components:* The kit may also contain other suitably packaged reagents and materials needed for the particular immunoassay protocol, including solid-phase matrices, if applicable, and standards.

The kits referred to above may include instructions for conducting the test. Furthermore, in preferred embodiments, the diagnostic kits are adaptable to high-throughput and/or automated operation.

Drug Targets and Screening Methods

According to the present invention, nucleotide sequences derived from the gene encoding a polymorphic form of ACE, AT1, AGT, renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, or β-adrenoceptor, and peptide sequences derived from that polymorphic for, are useful targets to identify cardiovascular drugs, *i.e.*, compounds that are effective in treating one or more clinical symptoms of cardiovascular disease. Drug targets include without limitation (i) isolated nucleic acids derived from the gene encoding ACE, AT1, AGT, renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, or β-adrenoceptor and (ii) isolated peptides and polypeptides derived from ACE, AT1, AGT, renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, or β-adrenoceptor polypeptides, each of which comprises one or more polymorphic positions.

In vitro screening methods

In one series of embodiments, an isolated nucleic acid comprising one or more polymorphic positions is tested *in vitro* for its ability to bind test compounds in a sequence-specific manner. The methods comprise:

(i) providing a first nucleic acid containing a particular sequence at a polymorphic position and a second nucleic acid whose sequence is identical to that of the first nucleic acid except for a different sequence at the same polymorphic position;

(ii) contacting the nucleic acids with a multiplicity of test compounds under conditions appropriate for binding; and

(iii) identifying those compounds that bind selectively to either the first or second nucleic acid sequence.

Selective binding as used herein refers to any measurable difference in any parameter of binding, such as, *e.g.*, binding affinity, binding capacity, *etc.*

In another series of embodiments, an isolated peptide or polypeptide comprising one or more polymorphic positions is tested *in vitro* for its ability to bind test compounds in a sequence-specific manner. The screening methods involve:

(i) providing a first peptide or polypeptide containing a particular sequence at a polymorphic position and a second peptide or polypeptide whose sequence is identical to the first peptide or polypeptide except for a different sequence at the same polymorphic position;

(ii) contacting the polypeptides with a multiplicity of test compounds under conditions appropriate for binding; and

(iii) identifying those compounds that bind selectively to one of the nucleic acid sequences.

In preferred embodiments, high-throughput screening protocols are used to survey a large number of test compounds for their ability to bind the genes or peptides disclosed above in a sequence-specific manner.

Test compounds are screened from large libraries of synthetic or natural compounds. Numerous means are currently used for random and directed synthesis of saccharide, peptide, and nucleic acid based compounds. Synthetic compound libraries are commercially available from Maybridge Chemical Co. (Trevillet, Cornwall, UK), Comgenex (Princeton, NJ), Brandon Associates (Merrimack, NH), and Microsource (New Milford, CT). A rare chemical library is available from Aldrich (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available from *e.g.* Pan Laboratories (Bothell, WA) or MycoSearch (NC), or are readily producible. Additionally, natural and synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means.

In vivo screening methods

Intact cells or whole animals expressing polymorphic variants of a gene encoding ACE, AT1, AGT, renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, or β-adrenoceptor can be used in screening methods to identify candidate cardiovascular drugs.

In one series of embodiments, a permanent cell line is established from an individual exhibiting a particular polymorphic pattern. Alternatively, cells (including without limitation mammalian, insect, yeast, or bacterial cells) are programmed to express a gene comprising one or more polymorphic sequences by introduction of appropriate DNA. Identification of candidate compounds can be achieved using any suitable assay, including without limitation (i) assays that measure selective binding of test compounds to particular polymorphic variants of the gene; (ii) assays that measure the ability of a test compound to modify (*i.e.*, inhibit or enhance) a measurable activity or function of the gene; and (iii) assays that measure the ability of a compound to modify (*i.e.*, inhibit or enhance) the transcriptional activity of sequences derived from the promoter (*i.e.*, regulatory) regions the gene.

In another series of embodiments, transgenic animals are created in which (i) a human *ACE, AT1, AGT, renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor*, or *β-adrenoceptor* gene having different sequences at particular polymorphic positions are stably inserted into the genome of the transgenic animal; and/or (ii) the endogenous genes are inactivated and replaced with human genes having different sequences at particular polymorphic positions. See, *e.g.*, Coffman, Semin. Nephrol., 1997, 17:404; Esther *et al.*, Lab. Invest., 1996, 74:953; Murakami *et al.*, Blood Press. Suppl., 1996, 2:36. Such animals can be treated with candidate compounds and monitored for one or more clinical markers of cardiovascular status.

Furthermore, populations that are not amenable to an established treatment for a cardiovascular disease or disorder can be selected for testing of alternative treatments. Moreover, treatments that are not as effective in the general population, but that are highly effective in the selected population, may be identified that otherwise would be overlooked. This is an especially powerful advantage of the present invention, since it eliminates some of the randomness associated with clinical trials.

The following are intended as non-limiting examples of the invention.

Example 1: <u>Methods for Identification of Polymorphic Positions in Human Genes Encoding ACE, AGT, and AT1</u>

The following studies were performed to identify polymorphic residues within the genes encoding human ACE, AGT, and AT1.

DNA samples were obtained from 277 individuals. The individuals were Caucasian males born in Uppsala, Sweden between 1920 and 1924. Individuals were selected for the test population based on their medical history, *i.e.*, they were either (i) healthy, with no signs of cardiovascular disease (100); or (ii) had suffered one of acute myocardial infarction (68), silent myocardial infarction (34), stroke (18), stroke and acute myocardial infarction (19), or high blood pressure at age 50 (39). DNA samples were obtained from each individual.

DNA sequence analysis was carried out by: (i) amplifying short fragments of each of the ACE, AGT, and AT1 genes using polymerase chain reaction (PCR) and (ii) sequencing the amplified fragments. The sequences obtained from each individual were then compared with known ACE, AGT, and AT1 genomic sequences (see Table 1).

(i) Amplification: PCR reactions employed the primers shown in Table 2 below.

Table 2 - PCR Primers

Name	Sequence	SEQ ID NO	Modification *)	Nucleotides	Numbering according to **)
ACE/79RB	5'-TGCGTGCTTCAGAAGTCC-3'	1	B	158-175	i+20: 1-175
ACE/82RB	5'-CCAGGGAGGTGAAGAAATC-3'	2	B	35-53	e20, J04144
ACE/84FT	5'-AGCCAGGCAGTAATGACCT-3'	3	T	1-19	i-19: 1-218
ACE/94FB	5'-GCCCACTGTTCCCTTATG-3'	4	B	1-18	i-21: 1-76
ACE/95RB	5'-TGCCCTGACTGACAGAGC-3'	5	B	105-122	i+23: 1-122
ACE/96RT	5'-GCCCTGGTGTGCCTGT-3'	6	T	1-16	i-22: 1-65
ACE/107F	5'-TGCCTGGATATGTGTTGC-3'	7	-	1-18	i-15: 1-225
ACE/107FB	5'-TGCCTGGATATGTGTTGC-3'	8	B	1-18	i-15: 1-225
ACE/108RB	5'-GCCCTCGCCTCTCACT-3'	9	B	23-38	i+16: 1-38
ACE/111RT	5'-TCCCCTCTCCCTGTACCT-3'	10	T	17-34	i+15: 1-34
ACE/114RB	5'-GTGCTGGGGTAGGGTAGA-3'	11	B	101-118	i+7: 1-118
ACE/118FT	5'-TCCCCCTGACCTGGCT-3'	12	T	221-236	i-7: 1-253
ACE/119FB	5'-GGGGCACCGTGATGTT-3'	13	B	1-16	i-4: 1-120
ACE/119FT	5'-GGGGCACCGTGATGTT-3'	14	T	1-16	i-4: 1-120
ACE/120RB	5'-GCCAGAGCCTTTGGTTT-3'	15	B	230-246	i+5: 1-246
ACE/122FB	5'-TGGAAGAGCCGACTTACAG-3'	16	B	1-19	i-5: 1-78
ACE/123RB	5'-TCCCAGAGGCAAAGAGG-3'	17	B	225-241	i+4: 1-241
ACE/130F	5'-GTTTCTACTGCGGCTTCAT-3'	18	-	1-19	i-8: 1-131
ACE/130FB	5'-GTTTCTACTGCGGCTTCAT-3'	19	B	1-19	i-8: 1-131

Name	Sequence	SEQ ID NO	Modification *)	Nucleotides	Numbering according to **)
ACE/134RB	5'-TCCTGGAAGAGGGAGTTTC-3'	20	B	148-166	i+9: 1-166
ACE/145F	5'-GCAGGATGAGAGCAACAAC-3'	21	-	1-18	i-7: 1-253
ACE/146F	5'-CTGGAGACCACTCCCATCCTTTCT-3'	22	-	1-24	i-17: 1-454
ACE/147R	5'-GATGTGGCCATCACATTCGTCAGAT-3'	23	-	1-25	e17, J04144
ACE/170RT	5'-CTTCCGTGGGACTCATGT-3'	24	T	23-40	i+5: 1-246
ACE/171RT	5'-TGCACCGTGAGGCTCTA-3'	25	T	136-152	i+8: 1-152
ACE/173F	5'-GCCCAATAGGAGGAAGCA-3'	26	MT	1-10, 1-9	i-2: 1-10, e2
ACE/174R	5'-CCCACCCCATCTCCAAGAA-3'	27	-	166-184	i-2: 1-184
ACE/175FB	5'-GCC-3'		MT, B	1-3	i-2: 1-10
ACE/176RT	5'-TCCCTGATGGGCTGCTCTC-3'	28	T	65-83	i-2: 1-184
ACE/177FT	5'-CAAGGCCCTCAACCAACTC-3'	29	T	1-19	i-24: 1-50
ACE/178RB	5'-TTCCCACAAAAGCTCCAGTG-3'	30	B	71-90	i+24: 1-108
ACE/179R	5'-GGCTCAAAATGGCAAGTGTT-3'	31	-	89-108	i+24: 1-108
ACE/180FT	5'-GGGCCATGTCCTTCTGACTC-3'	32	T	1-20	i-25: 1-45
ACE/181RB	5'-CAGCCTGGAGGGGTTAAGA-3'	33	B	33-51	i+25: 1-51
ACE/182R	5'-CCCTTCTGAGCGAGCTGAGT-3'	34	-	1-6,1-14	i-26: 1-6, e26, J04144
ACE/183F	5'-GGCCATGTTGAGCTACTTCAA-3'	35	-	83-103	e25, J04144
ACE/184FB	5'-CCTCCAGCCTTGGGTCTTAA-3'	36	B	19-38	i+25: 1-38
ACE/185RT	5'-TTCCCATCCCAGTCTCTGGT-3'	37	T	269-288	e26, J04144
ACE/188RT	5'-GGCAGCCTGGTTGATGAGT-3'	38	T	116-134	e17, J04144

Name	Sequence	SEQ ID NO	Modification *)	Nucleotides	Numbering according to **)
ACE/192FB	5'-ATTCCAGCTCTGAAATTCTCTGA-3'	39	B	1-23	i-17: 1-85
ACP/3FT	5'-GAGCCCCTCCAGCACCTC-3'	40	T	499-5017	X94359
ACP/4RB	5'-ACCCGAGCCTGCCCACC-3'	41	B	5302-5318	X94359
ACP/5FT	5'-GGTCGGGCTGGGAAGATC-3'	42	T	5232-5249	X94359
ACP/6RB	5'-TCGGCTCTGCCCCTTCTC-3'	43	B	5576-5593	X94359 + additional downstream sequence
ACP/7FT	5'-GCCCTTTCTCCAGCTTCCTCT-3'	44	T	5361-5381	X94359
ACP/8RB	5'-CGGCGGCAGCAGCAACA-3'	45	B	5666-5682	X94359 + additional downstream sequence
ACP/11FB	5'-GAGCCCCTCCAGCACCTC-3'	46	B	499-5017	X94359
ACP/12RT	5'-ACCCGAGCCTGCCCACC-3'	47	T	5302-5318	X94359
ACP/13FB	5'-GGTCGGGCTGGGAAGATC-3'	48	B	5232-5249	X94359
ACP/14RT	5'-TCGGCTCTGCCCCTTCTC-3'	49	T	5576-5593	X94359 + additional downstream sequence
ACP/15FB	5'-GCCCTTTCTCCAGCTTCCTCT-3'	50	B	5361-5381	X94359

Name	Sequence	SEQ ID NO	Modification *)	Nucleotides	Numbering according to **)
ACP/16RT	5'-CGGCGGCAGCAGCAACA-3'	51	T	5666-5682	X94359 + additional downstream sequence
ANG/1FT	5'-ATGGCACTTAAAGGTCAGTTAAT-3'	52	T	336-358	M24686
ANG/2RB	5'-TACGGAAGCCCAAGAAGTT-3'	53	B	726-745	M24686
ANG/5FT	5'-CTCCCCAACGGCTGTCTT-3'	5 4	T	797-814	M24686
ANG/6RB	5'-AGCAGCAACATCCAGTTCTGT-3'	55	B	1119-1139	M24686
ANG/7FT	5'-TCCCACGCTCTCTGGACTT-3'	56	T	1099-1117	M24686
ANG/8RB	5'-CTGATCTCAGCTACACATGGATACTA-3'	57	B	1290-1315	M24686
ANG/15FT	5'-CCTGTCTTGGGTGACTCTTC-3'	58	T	7-26	M24687
ANG/17FB	5'-TTCTGGGCTAAATGGTGACA-3'	59	B	285-304	M24686
ANG/18RT	5'-CTTGTCTTCGGTGTCAAGTTT-3'	60	T	675-695	M24686
ANG/19FB	5'-GGGAGCCTTGGACCACAC-3'	61	B	839-856	M24686
ANG/20RT	5'-AGCCTGCATGAACCTGTCAA-3'	62	T	1147-1167	M24686
ANG/21FB	5'-TGGTGGGCGTGTTCACA-3'	63	B	1018-1034	M24686
ANG/22RT	5'-GCCAGAGCCAGCAGAGA-3'	64	T	1264-1280	M24686
ANG/29RB	5'-CCACATTCCAGGGGAGAC-3'	65	B	335-352	M24687
ANG/30FB	5'-CCTGTCTTGGGTGACTCTTC-3'	66	B	7-26	M24687
ANG/32RT	5'-CCACATTCCAGGGGAGAC-3'	67	T	334-352	M24687
ANP/1FT	5'-GTCCCTTCAGTGCCCTAATAC-3'	68	T	314-334	X15232

Name	Sequence	SEQ ID NO	Modification *)	Nucleotides	Numbering according to **)
ANP/2RB	5'-ACAGCCAGATTGAAAGACACA-3'	69	B	593-613	X15232
ANP/3FT	5'-AACCCTTTTACTGGTCATGTGA-3'	70	T	492-513	X15232
ANP/4RB	5'-CGCTCATGGGATGTGTGAC-3'	71	B	747-765	X15232
ANP/5FT	5'-TGTTTTCCCCAGTGTCTATTAGA-3'	72	T	686-708	X15232
ANP/6RB	5'-GCAGGGTCGAGTTACACATTT-3'	73	B	982-1003	X15232
ANP/7FT	5'-CCTCAGGCTGTCACACACCTA-3'	74	T	909-929	X15232
ANP/8RB	5'-CGGCTTACCTTCTGCTGTAGT-3'	75	B	1246-1266	X15232
ANP/9FB	5'-CTCCTTGAACCTGCTTGTGTT-3'	76	B	273-293	X15232
ANP/10RT	5'-GCATTGAAAGATGTGCTGTTCT-3'	77	T	548-569	X15232
ANP/11FB	5'-TAACGACTACAAAAGCAAGTCTTAC-3'	78	B	446-469	X15232
ANP/12RT	5'-AGAGGGCAGGGGAGAGTCT-3'	79	T	805-823	X15232
ANP/13FB	5'-GGCAGCAGGGTCAGAAGT-3'	80	B	766-783	X15232
ANP/14RT	5'-GCTGGAGAGGAGGGTTACAT-3'	81	T	1127-1146	X15232
ANP/15FB	5'-TGCAAACTTCGGTAAATGTGT-3'	82	B	970-990	X15232
ANP/16RT	5'-CAGAACAACGGCAGCTTCT-3'	83	T	1224-1242	X15232
AT1/5FT	5'-ACTGGCTGACTTATGCTTTTTACT-3'	84	T	547-570	S77410
AT1/6RB	5'-GGGTTGAATTTTGGGACTCATA-3'	85	B	884-905	S77410
AT1/7FT	5'-GCCAGTTTGCCAGCTATAAT-3'	86	T	809-828	S77410
AT1/8RB	5'-TGATGCCTAGTTGAATCAATACA-3'	87	B	1123-1145	S77410
AT1/9FT	5'-GAAGGCTTATGAAATTCAGAAGA-3'	88	T	1003-1025	S77410
AT1/10RB	5'-AAAGTCGGTTCAGTCCACATAA-3'	89	B	1535-1556	S77410

Name	Sequence	SEQ ID NO	Modification *)	Nucleotides	Numbering according to **)
AT1/16FB	5'-AAACAGCTTGGTGGTGATAGTC-3'	90	B	469-490	S77410
AT1/17RT	5'-GCAGGTGACTTTGGCTACAA-3'	91	T	762-781	S77410
AT1/18FB	5'-CCTGTACGCTAGTGTGTTTCTACT-3'	92	B	667-690	S77410
AT1/19RT	5'-AGGAAACAGGAAACCCAGTATAT-3'	93	T	932-955	S77410
AT1/22FB	5'-CTGGATTCCCCACCAAATAT-3'	94	B	1090-1109	S77410
AT1/23RT	5'-TGCTCCTTCTTTCACAAAATTAC-3'	95	T	1438-1460	S77410
ATP/1FT	5'-CTTCCGTTATTATGTGTGATATTAGT-3'	96	T	1244-1269	U07144
ATP/2RB	5'-GCATGTACCTAAAAAGTCCTGTC-3'	97	B	1566-1588	U07144
ATP/5FT	5'-ATTGGCATATCCATCACCTTAA-3'	98	T	1628-1649	U07144
ATP/6RB	5'-GATCTCCCAACTCATGCTATGA-3'	99	B	1961-1982	U07144
ATP/7FT	5'-ATTGGATTCAATTTGCCTACAT-3'	100	T	1846-1867	U07144
ATP/8RB	5'-TTTGGTAATACAGTTGTGGATCATA-3'	101	B	2159-2184	U07144
ATP/9FT	5'-TGCAACTTGGGTAGCATGTC-3'	102	T	2077-2096	U07144
ATP/10RB	5'-AGTCGTCCCGTGTCAACTATC-3'	103	B	2370-2390	U07144
ATP/11FB	5'-CGTTGTCTTCCGTTATTATGTGT-3'	104	B	1238-1260	U07144
ATP/12RT	5'-TTATTGCATGTACCTAAAAAGTGTA-3'	105	T	1455-1479	U07144
ATP/15FB	5'-GCATTCATATAAAGATCAAATCAGT-3'	106	B	1600-1624	U07144
ATP/16RT	5'-CACCCTGATAACAAAACCAGATA-3'	107	T	1929-1951	U07144
ATP/17FB	5'-CTTTCTGGCATCAACCTCACT-3'	108	B	1794-1814	U07144
ATP/18RT	5'-ACTTTTAAGGACGAATTAGAGAACT-3'	109	T	2214-2238	U07144
ATP/19FB	5'-GTCCACCCTTGAATTTCATAAC-3'	110	B	2115-2136	U07144

Name	Sequence	SEQ ID NO	Modification *)	Nucleotides	Numbering according to **)
ATP/20RT	5'-CCCAACCTCCTCCCTCTC-3'	111	T	2396-2413	U07144
ATP/21FT	5'-GCTCGCTCTCCCTCACGAC-3'	112	T	2310-2328	U07144
ATP/22RB	5'-TCCAGCCGCTCCCCATC-3'	113	B	2657-2673	U07144
ATP/23FB	5'-GCTCGCTCTCCCTCACGAC-3'	114	B	2310-2328	U07144
ATP/24RT	5'-TCCAGCCGCTCCCCATC-3'	115	T	2657-2673	U07144
ATR/1F	5'-GCCCCTCAGATAATGTAAGCTC-3'	116	-	1353-1374	S77410
ATR/2R	5'-AACCGGCACGAAAACTTTACT-3'	117	-	1834-1854	S77410
ATR/3aF	5'-GCACTTCACTACCAAATGAGCA-3'	118	-	1476-1500	S77410
ATR/4cF	5'-GCACTTCACTACCAAATGAGCC-3'	119	-	1476-1500	S77410

Where indicated, the primers were modified in one of the following ways: (i) a biotin molecule was conjugated to the 5' terminus of the indicated sequence (B); (ii) a sequence of nucleotides derived from M13, 5'-CAGGAAACAGCTATGACT-3' (SEQ ID NO:120), was added at the 5' terminus of the indicated sequence (MT); or (iii) the sequence 5'-AGTCACGACGTTGTAAAACGACGGCCAGT-3' (SEQ ID NO:121) was added at the 5' terminus of the indicated sequence (T = Tail). Nucleotides were numbered according to the GenBank sequences listed in Table 1 where indicated. When the sequences involved were not publicly available, the numbering was as in the following examples: The designation "i-4: 1-200" indicates that the primer sequence is located within the sequence extending 200 bp upstream of, and including, the nucleotide immediately upstream of the first coding nucleotide of exon 4. Similarly, the designation "i+4: 1-200" indicates that the primer sequence is located within the sequence extending from the nucleotide that is located immediately downstream of the last coding nucleotide of exon 4 downstream for 200 bp. In each case, the specific location of the primer sequence is indicated in Table 2 in the column marked "Nucleotides".

The reaction components used for PCR are described in Table 3, and the reaction conditions for PCR are described Table 4, below.

Table 3 -PCR Components

Condition	Components	Volume
A	Ultrapure dNTP Set 2.5 mM (dATP:dCTP:dGTP:dTTP = 1:1:1:1), (Pharmacia Biotech)	4 µl
	10xPCR buffer II, (Perkin Elmer)	5 µl
	$MgCl_2$ solution 2.5 mM,	3 µl
	AmpliTaq® DNApolymerase(Perkin Elmer) (5U/ml)	0.15 µl
	Primer 1	1 µl
	Primer 2	1 µl
	DNA solution	1 µl
	R/O-purified water q.s.	Tot. 50 µl
B	Ultrapure dNTP Set 2.5 mM (dATP:dCTP:dGTP:dITP:dTTP = 2:2:1:1:2), (Pharmacia Biotech)	4 µl
	10xPCR buffer II, (Perkin Elmer)	5 µl
	$MgCl_2$ solution 2.5 mM, (Perkin Elmer)	3 µl
	AmpliTaq® DNApolymerase (5U/ml)	0.15 µl
	Primer 1	1 µl
	Primer 2	1 µl
	DNA solution	1 µl
	R/O-purified water q.s.	Tot. 50 µl
C	Ultrapure dNTP Set 2.5 mM (dATP:dCTP:dGTP:dITP:dTTP = 4:4:1:3:4), (Pharmacia Biotech)	4 µl
	10xPCR buffer II, (Perkin Elmer)	5 µl

Condition	Components	Volume
	$MgCl_2$, solution 2.5 mM, (Perkin Elmer)	3 µl
	AmpliTaq® DNApolymerase (5U/ml)	0.15 µl
	Primer 1	1 µl
	Primer 2	1 µl
	DNA solution	1 µl
	R/O-purified water q.s.	Tot. 50 µl
D	Ultrapure dNTP Set 2.5 mM (dATP:dCTP:dGTP:dITP:dTTP = 6:6:1:5:6), (Pharmacia Biotech)	4 µl
	10xPCR buffer II, (Perkin Elmer)	5 µl
	$MgCl_2$, solution 2.5 mM, (Perkin Elmer)	3 µl
	AmpliTaq® DNApolymerase (5U/ml)	0.15 µl
	Primer 1	1 µl
	Primer 2	1 µl
	DNA solution	1 µl
	R/O-purified water q.s.	Tot. 50 µl
E	Ultrapure dNTP Set 2.5 mM (dATP:dCTP:dGTP:dITP:dTTP = 4:4:1:3:4), (Pharmacia Biotech)	4 µl
	10xPCR buffer II, (Perkin Elmer)	5 µl
	$MgCl_2$, solution 2.5 mM, (Perkin Elmer)	2.5 µl
	DMSO	2.5 µl

Condition	Components	Volume
	AmpliTaqGold® DNApolymerase (5U/ml)	0.5 µl
	Primer 1	1 µl
	Primer 2	1 µl
	DNA solution	1 µl
	R/O-purified water q.s.	Tot. 50 µl
F	Ultrapure dNTP Set 2.5 mM (dATP:dCTP:dGTP:dTTP = 1:1:1:1) (Pharmacia Biotech)	4 µl
	10xPCR buffer II, (Perkin Elmer)	5 µl
	MgCl, solution 2.5 mM, (Perkin Elmer)	2 µl
	AmpliTaq® DNApolymerase (5U/ml)	0.5 µl
	Primer 1	1 µl
	Primer 2	1 µl
	DNA solution	1 µl
	R/O-purified water q.s.	Tot. 50 µl
G	Ultrapure dNTP Set 2.5 mM (dATP:dCTP:dGTP:dTTP = 1:1:1:1) (Pharmacia Biotech)	4 µl
	10xPCR buffer II, (Perkin Elmer)	5 µl
	MgCl, solution 2.5 mM, (Perkin Elmer)	2 µl

Condition	Components	Volume
	AmpliTaq® DNApolymerase (5U/ml)	0.5 µl
	Primer 1	1 µl
	Primer 2	1 µl
	DNA solution	1 µl
	R/O-purified water q.s.	Tot. 50 µl
H	Ultrapure dNTP Set 2.5 mM (dATP:dCTP:dGTP:dITP:dTTP = 4:4:1:3:4), (Pharmacia Biotech)	4 µl
	10xPCR buffer II, (Perkin Elmer)	5 µl
	$MgCl_2$ solution 2.5 mM, (Perkin Elmer)	4 µl
	AmpliTaqGold® DNApolymerase (5U/ml)	0.5 µl
	Primer 1	1 µl
	Primer 2	1 µl
	DNA solution	1 µl
	R/O-purified water q.s.	Tot. 50 µl

Table 4 - PCR Reaction Conditions

PCR-method	Temperature*)	Time *)	Temperature **)	Time	Temperature	Time	No of cycles ***)
25	94	15 s	55	30 s	72	45 s	35
	72	5 min					1
	22	∞					
27	94	15 s	55	30 s	72	45 s	35
	72	5 min					1
	22	∞					
36	94	2 min					1
	94	15 s	58	30 s	72	45 s	35
	72	5 min					1
	22	∞					
38	94	2 min					1
	94	15 s	60	30 s	72	45 s	15
	72	5 min					1
	22	∞					
40	94	2 min					1
	94	15 s	60	30 s	72	45 s	35
	72	5 min					1
	22	∞					

PCR-method	Temperature*)	Time *)	Temperature **)	Time	Temperature	Time	No of cycles ***)
54	96	5 min					1
	96	30 s	61	30 s	72	45 s	15
	72	5 min					1
	22	∞					
56	96	5 min					1
	96	30 s	61	30 s	72	45 s	35
	72	5 min					1
	22	∞					
64	95	2 min					
	95	15 s	59	30 s	72	45 s	40
	72	5 min					1
	22	∞					
70	95	5 min					
	95	15 s	59	30 s	72	45 s	50
	72	5 min					1
	22	∞					

All temperatures are given in degrees Celsius.

*) indicates the default initial temperatures (°C) and times of the program.

**) indicates the default temperature (°C) of the program.

***) indicates the default number of cycles of the program, referring to the section of the PCR program where three different temperatures are employed.

Any differences are indicated in "Modifications" in Table 5 below.

The amplified fragments are described in Table 5 below with respect to the primers and PCR reaction conditions used for amplification.

Table 5 - Amplified Fragments

Fragment	Primer 1	Primer 2	PCR method	Modifications of PCR method	PCR reaction conditions
ANPf1F	ANP/1FT	ANP/2RB	64		A
ANPf2F	ANP/3FT	ANP/4RB	64		B
ANPf3F	ANP/5FT	ANP/6RB	64	anneal. temp: 48°C	A
ANPf4F	ANP/7FT	ANP/8RB	64	anneal. temp: 59°C	D
ANPf5R	ANP/9FB	ANP/10RT	64		A
ANPf6R	ANP/11FB	ANP/12RT	64		B
ANPf7R	ANP/13FB	ANP/14RT	64		A
ANPf8R	ANP/15FB	ANP/16RT	64		C
ANGe2f1F	ANG/1FT	ANG/2RB	64		C
ANGe2f3F	ANG/5FT	ANG/6RB	64		C
ANGe2f4F	ANG/7FT	ANG/8RB	64		A
ANGe2f5R	ANG/17FB	ANG/18RT	64		A
ANGe2f7R	ANG/19FB	ANG/20RT	64		A
ANGe2f8R	ANG/21FB	ANG/22RT	64		A
ANGe3F	ANG/15FT	ANG/29RB	64	anneal. temp: 57°C	F
ANGe3R	ANG/30FB	ANG/32RT	64	anneal. temp: 57°C, 45 cycles	A

Fragment	Primer 1	Primer 2	PCR method	Modifications of PCR method	PCR reaction conditions
ACPf2F	ACP/3FT	ACP/4RB	70	anneal. temp: 62°C	E
ACPf3F	ACP/5FT	ACP/6RB	70	anneal. temp: 58°C	E
ACPf4F	ACP/7FT	ACP/8RB	70		E
ACPf6R	ACP/11FB	ACP/12RT	70	anneal. temp: 62°C	E
ACPf7R	ACP/13FB	ACP/14RT	70	anneal. temp: 58°C	E
ACPf8R	ACP/15FB	ACP/16RT	70		E
ACEe2R PCR1	ACE/173F	ACE/174R	38		A
ACEe2R PCR2	ACE/175FB	ACE/176RT	40		A
ACEe4F PCR1	ACE/119FB	ACE/120RB	27		A
ACEe4F PCR2	ACE/119FT	ACE/123RB	25		A
ACEe5R PCR1	ACE/119FB	ACE/120RB	27		A
ACEe5R PCR2	ACE/122FB	ACE/170RT	25		A
ACEe7F PCR1	ACE/145F	ACE/114RB	27		A
ACEe7F PCR2	ACE/118FT	ACE/114RB	25		A
ACEe8R PCR1	ACE/130F	ACE/134RB	27		A
ACEe8R PCR2	ACE/130FB	ACE/171RT	25		A
ACEe15R PCR1	ACE/107F	ACE/108RB	27		A
ACEe15R PCR2	ACE/107FB	ACE/111RT	25		A
ACEe17R	ACE/192FB	ACE/188RT	40	anneal. temp: 63°C, 40 cycles	A
ACEe19F PCR1	ACE/84FT	ACE/79RB	27		A
ACEe19F PCR2	ACE/84FT	ACE/82RB	25		A
ACEe21R PCR1	ACE/94FB	ACE/95RB	27		A
ACEe21R PCR2	ACE/94FB	ACE/96RT	25		A

Fragment	Primer 1	Primer 2	PCR method	Modifications of PCR method	PCR reaction conditions
ACEe24F PCR1	ACE/177FT	ACE/179R	38		A
ACEe24F PCR2	ACE/177FT	ACE/178RB	40		A
ACEe25F PCR1	ACE/180FT	ACE/182R	38		A
ACEe25F PCR2	ACE/180FT	ACE/181RB	40		A
ACEe26R PCR1	ACE/183F	ACE/185RT	54		A
ACEe26R PCR2	ACE/184FB	ACE/185RT	56		A
ACEDI	ACE/146F	ACE/147R	36		A
ATPf1F	ATP/1FT	ATP/2RB	64		A
ATPf3F	ATP/5FT	ATP/6RB	64	anneal. temp: 58°C	A
ATPf4F	ATP/7FT	ATP/8RB	64	anneal. temp: 48°C	A
ATPf5F	ATP/9FT	ATP/10RB	64	anneal. temp: 58°C	A
ATPf6R	ATP/11FB	ATP/12RT	64	anneal. temp: 48°C	A
ATPf8R	ATP/15FB	ATP/16RT	64	anneal. temp: 55°C	G
ATPf9R	ATP/17FB	ATP/18RT	64	anneal. temp: 54°C	A
ATPf10R	ATP/19FB	ATP/20RT	64		A
ATPf11F	ATP/21FT	ATP/22RB	64	initial denaturation: 95°C, 12 min.	H
ATPf12R	ATP/23FB	ATP/24RT	64	initial denaturation: 95°C, 12 min.	H
AT1e5f2F	AT1/5FT	AT1/6RB	64		A
AT1e5f3F	AT1/7FT	AT1/8RB	64		A
AT1e5f4F	AT1/9FT	AT1/10RB	64		C
AT1e5f6R	AT1/16FB	AT1/17RT	64		A
AT1e5f7R	AT1/18FB	AT1/19RT	64		C
AT1e5f9R	AT1/22FB	AT1/23RT	64		A

Fragment	Primer 1	Primer 2	PCR method	Modifications of PCR method	PCR reaction conditions
AT1-spec. 1	ATR/1F ATR/3aF	ATR/2R	40	anneal. temp: 63°C	A
AT1-spec. 2	ATR/1F ATR/2R	ATR/4cF	40	anneal. temp: 63°C	A

All of the PCR products (except fragments ACEDI, AT1-spec. 1 and AT1-spec. 2) were subjected to solid phase sequencing according to the protocol commercially available from Pharmacia Biotech. The sequencing reactions are performed with a sequencing primer having a complementary sequence to the "Tail" sequence previously described in Table 2. The nucleotide sequence of the sequencing primer was 5'-CGACGTTGTAAAACGACGGCCAGT-3' (SEQ ID NO:122), and the primer was fluorescently labeled with a Cy-5-molecule on the 5'-nucleotide. The positions carrying a genetic variation were identified by determination of the nucleotide sequence by the use of the ALFexpress™ system commercially available from Pharmacia Biotech.

The detection of the fragment ACEDI was performed by analyzing the sizes of the amplified fragments by gel electrophoresis, where the presence of a shorter PCR product (192 base pairs) indicated the D-allele and a longer PCR product (479 base pairs) indicated the I-allele. The presence of both bands indicated a heterozygote for the two alleles. The detection of the allele-specific reaction of position AT1-1271 was performed by separately running two parallel PCR reactions on the same sample and comparing the sizes of the amplified fragments. A PCR product of 501 base pairs should always be present as a control in both parallel runs, whereas the presence of a PCR product of 378 base pairs in the reaction designated AT1-spec. 1 indicated the presence of an A in this position. The presence of a PCR product of 378 base pairs in the reaction designated AT1-spec. 2 indicated a C in this position. If the shorter PCR product was present in both reactions, the individual is a heterozygote for A and C.

Results

The analysis described above resulted in the identification of polymorphic positions within the regulatory and coding/intron segments of the human genes encoding ACE, AGT, and AT1. The polymorphic positions, the variant nucleotides found at each of the positions, and the PCR fragment in which the polymorphism was identified are shown in Table 6 below. Also shown are the frequencies of each genotype in a population of 90 individuals, expressed as the percent of the study population having that genotype. Polymorphisms that resulted in alternate amino acids in ACE, AGT, or AT1 are also indicated. As used herein below, the designations "AGR" , "ACR", and "ATR" refer to the regulatory regions of the human AGT, ACE, and AT1 genes, respectively; and the designations "AGT", "ACE", and "AT1", refer to the coding regions of the AGT, ACE, and AT1 genes.

Table 6 - Polymorphisms Found in ACE, AGT, and AT1 Genes

Gene	Position	Reported genotype	Genetic variation	Frequency (per cent)	Amino acid change	Fragment	Reference (if any)
AGR	395	T	TT-TA-AA	88-11-1	None	ANPf1F ANPf5R	-
AGR	412	C	CC-CT	99-1	None	ANPf1F ANPf5R	-
AGR	432	G	GG-GA	81-19	None	ANPf1F ANPf5R	-
AGR	449	C	TT-TC	92-8	None	ANPf1F ANPf5R	-
AGR	692	C	CC-CT	81-19	None	ANPf2F ANPf6R	-
AGR	839	G	GG-GA	93-7	None	ANPf3F ANPf7R	-
AGR	1007	G	GG-GA	81-19	None	ANPf4F ANPf7R	-
AGR	1072	G	GG-GA	89-11	None	ANPf4F ANPf7R	-
AGR	1204	C	CC-CA-AA	67-33	None	ANPf4F ANPf8R	-
AGR	1218	A	AA-AG-GG	14-55-31	None	ANPf4F ANPf8R	Inuoe, I et. al. J. C. I., 1997, 99: 1786-1789.
AGT	273	C	CC-CT	99-1	None	ANGe2f1F ANGe2f5R	-
AGT	620	C	CC-CT	80-20	Thr - Met	ANGe2f3F ANGe2f7R	JeunmaîtreX, *et al.* Cell, 1992, 71:169-180.
AGT	803	T	TT-TC-CC	35-52-13	Met - Thr	ANGe2f4F ANGe2f8R	JeunmaîtreX, *et al.* Cell, 1992, 71:169-180.

Gene	Position	Reported genotype	Genetic variation	Frequency (per cent)	Amino acid change	Fragment	Reference (if any)
AGT	912	C	CC-CT	99-1	None	ANGe3F ANGe3R	-
AGT	997	G	CC	100	Glu - Gln	ANGe3F ANGe3R	-
AGT	1116	G	GG-GA-AA	87-12-1	None	ANGe3F ANGe3R	-
AGT i3	49 Numbering according to GenBank entry M24688	A	AA-AG	80-20	None	ANGe3F ANGe3R	-
AGT	1174	C	CC-CA	99-1	Leu - Met	ANGe4F ANGe4R	-
ACR	5106	C	CC-CT	98-2	None	ACPf2F ACPf6R	-
ACR	5349	A	AA-AT-TT	35-46-19	None	ACPf3F ACPf7R	Villard, E. *et al.* Am. J. Hum. Genet., 1996, 58: 1268-1278
ACR	5496	T	TT-TC-CC	35-46-19	None	ACPf4F ACPf8R	Villard, E. *et al.* Am. J. Hum. Genet., 1996, 58: 1268-1278
ACE	375	A	CC	100	None	ACEe2R	-
ACE	582	C	CC-CT	94-6	None	ACEe4F	-
ACE	731	A	AA-AG	96-4	Tyr - Cys	ACEe5R	-
ACE	1060	G	GG-GA	97-3	Gly - Arg	ACEe7F	-
ACE	1215	C	CC-CT-TT	35-42-23	None	ACEe8R	Villard, E. *et al.* Am. J. Hum. Genet., 1996, 58: 1268-1278
ACE	2193	G	GG-GA-AA	20-57-23	None	ACEe15R	Villard, E. *et al.* Am. J. Hum. Genet.,1996, 58: 1268-1278

Gene	Position	Reported genotype	Genetic variation	Frequency (per cent)	Amino acid change	Fragment	Reference (if any)
ACE	1451		DD-DI-II	29-54-20	None	ACEDI	Villard, E. *et al.* Am. J. Hum. Genet., 1996, 58: 1268-1278
ACE	2328	A	AA-AG-GG	20-57-23	None	ACEe17R	Villard, E. *et al.* Am. J. Hum. Genet.,1996, 58: 1268-1278
ACE	2741	G	TT	100	Gly - Val	ACEe19F	-
ACE	3132	C	CC-CT	99-1	None	ACEe21R	-
ACE	3387	T	TT-TC-CC	20-57-23	None	ACEe24F	-
ACE	3503	C	GG	100	Ala - Gly	ACEe25F	-
ACE	3906	G	GG-GA	91-9	None	ACEe26R	-
ATR	1427	A	AA-AT-TT	77-22-1	None	ANPf1F ANPf6R	-
ATR	1756	T	TT-TA-AA	75-24-1	None	ANPf3F ANPf8R	-
ATR	1853	T	TT-TG-GG	82-11-1	None	ANPf3F ANPf8R	-
ATR	2046	T	CC-CT-TT	46-46-8	None	ANPf4F ANPf9R	-
ATR	2354	A	AA-AC-CC	73-26-1	None	ANPf5F ANPf10R	-
ATR	2355	G	GG-GC-CC	73-26-1	None	ANPf5F ANPf10R	-
ATR	2415	A	AA-AG	75-24-1	None	ANPf11F ANPf12R	-
AT1	449	G	GG-GC	99-1	Ser - Thr	AT1e5f2F AT1e5f6R	-
AT1	678	T	CC-CT-TT	31-48-21	None	AT1e5f3F AT1e5f7R	Rolfs A, et. al. Eur. Heart. J., 1994, 15: Suppl. D, 108-112.

Gene	Position	Reported genotype	Genetic variation	Frequency (per cent)	Amino acid change	Fragment	Reference (if any)
AT1	1167	A	AA-AG	92-8	None	AT1e5f4F AT1e5f9R	Rolfs A, et. al. Eur. Heart. J., 1994, 15: Suppl. D, 108-112.
AT1	1271	A	AA-AC-CC	50-40-10	None	AT1-spec	Bonnardeaux, A. *et al.* Hypertension, 1994, 24:63-69.

A subset of these polymorphic positions were further analyzed in an additional 187 individuals. Table 7 shows the polymorphic positions, the sequence at these positions, and the genotype frequencies for each position in a population of 277 as described in Example 1 above.

Table 7 - Polymorphic Positions and Frequencies

Gene	Position	Genetic variation	Frequency (per cent)
AGR	395	TT-TA-AA	87-12-7
AGR	432	GG-GA-AA	78-21-1
AGR	449	TT-TC-CC	94-5-1
AGR	692	CC-CT-TT	78-21-1
AGR	839	GG-GA	96-4
AGR	1007	GG-GA-AA	78-21-1
AGR	1072	GG-GA	76-24
AGR	1204	CC-CA-AA	3-27-70
AGR	1218	AA-AG-GG	16-50-34
AGT	620	CC-CT-TT	75-23-2
AGT	803	TT-TC-CC	34-50-16
AGT	1116	GG-GA-AA	83-15-2
ACR	5349	AA-AT-TT	37-44-19
ACR	5496	TT-TC-CC	38-43-19
ACE	1060	GG-GA	96-4
ACE	1215	CC-CT-TT	34-46-20
ACE	2193	GG-GA-AA	22-53-25
ACE	2328	AA-AG-GG	23-52-25
ACE	3387	TT-TC-CC	24-53-23
ACE	3906	GG-GA-AA	86-13-1
ATR	1427	AA-AT-TT	72-26-2
ATR	1756	TT-TA-AA	72-25-3
ATR	1853	TT-TG-GG	73-25-2
ATR	2046	CC-CT-TT	47-41-12
ATR	2354	AA-AC-CC	72-26-2
ATR	2355	GG-GC-CC	71-27-2

Gene	Position	Genetic variation	Frequency (per cent)
ATR	2415	AA-AG-GG	73-25-2
AT1	678	CC-CT-TT	26-51-23
AT1	1167	AA-AG	88-12
AT1	1271	AA-AC-CC	55-36-9

Example 2: **Correlation of Polymorphism Patterns with Cardiovascular Disease**

The polymorphic positions identified as in Example 1 were correlated with the following markers of cardiovascular status present in the study population: healthy (100 individuals evaluated); myocardial infarction (MI) (120 individuals); stroke (37 individuals); and high blood pressure (BP) (39 individuals). Polymorphic patterns, *i.e.*, combinations of sequences at particular polymorphic positions, that show a statistically significant correlation with one or more of these markers are shown below.

Table 8 - Polymorphism Patterns

ACR 5349 A/T, AGR 1218 A

	Healthy	MI	Stroke	High BP	Total (n)
# of events	3	7	3	5	17
% within group	3	5.8	8.1	12.8	

ACR 5496 C, AGR 1204 A/C

	Healthy	MI	Stroke	High BP	Total (n)
# of events	2	7	3	2	13
% within group	2	5.8	8.1	5.1	

ACR 5496 C/T, AGR 1218 A, AGT 620 C/T

	Healthy	MI	Stroke	High BP	Total (n)
# of events	4	13	1	3	21
% within group	4	10.8	2.7	7.7	

ACE 2193 A, AGR 1204 C, ACE 2328 G

	Healthy	MI	Stroke	High BP	Total (n)
# of events	0	11	3	3	16
% within group	0	9.2	8.1	7.7	

ACE 2193 A, AGR 1204 A/C

	Healthy	MI	Stroke	High BP	Total (n)
# of events	1	1	0	1	3
% within group	1	0.8	0	2.6	

ACE 3387 T, AGR 1218 A

	Healthy	MI	Stroke	High BP	Total (n)
# of events	2	4	1	3	10
% within group	2	3.3	2.7	7.7	

ACE 3387 T, AGT 620 C/T

	Healthy	MI	Stroke	High BP	Total (n)
# of events	1	10	3	2	15
% within group	1	8.3	8.1	5.1	

AGR 1204 A/C, AT1 678 C/T

	Healthy	MI	Stroke	High	Total (n)
# of events	5	23	5	6	37
% within group	5	19.2	13.5	15.4	

AGR 1204 A/C, AT1 1271 A/C

	Healthy	MI	Stroke	High BP	Total (n)
# of events	3	17	3	4	26
% within group	3	14.2	8.1	10.3	

ACE 1215 C, AGR 1204 A/C

	Healthy	MI	Stroke	High BP	Total (n)
# of events	3	13	5	6	25
% within group	3	10.8	13.5	15.4	

AGR 1204 A/C, AT1 1167 A, ACE 3906 A/G

	Healthy	MI	Stroke	High BP	Total (n)
# of events	0	5	1	0	6
% within group	0	4.2	2.7	0	

AGR 1204 A, AGT 620 C, AT1 1271 A, AT1 1167 A, AGR 395 A/T

	Healthy	MI	Stroke	High BP	Total (n)
# of events	1	4	5	3	11
% within group	1	3.3	13.5	7.7	

AGR 1204 A/C, AGT 620 C/T, AT1 1271 A/C, AT1 1167 A, AGR 395 T

	Healthy	MI	Stroke	High BP	Total (n)
# of events	3	13	3	2	20
% within group	3	10.8	8.1	5.1	

AGR 1204 A/C, AGT 620 C/T, AT1 1271 A/C, AT1 1167 A/G, AGR 395 T

	Healthy	MI	Stroke	High BP	Total (n)
# of events	0	2	0	1	3
% within group	0	1.7	0	2.6	

Summary of the three previous polymorphic patterns (which involve the same polymorphic positions):

	Healthy	MI	Stroke	High BP	Total (n)
# of events	4	19	8	6	34
% within group	4	15.8	21.6	15.4	

AGR 1204 A, AT1 678 C, AT1 1167 A, AGR 395 A/T

	Healthy October 12, 1999	MI	Stroke	High BP	Total (n)
# of events	1	2	2	1	5
% within group	1	1.7	5.4	2.6	

AGR 1204 A/C, AT1 678 C/T, AT1 1167 A, AGR 395 T

	Healthy	MI	Stroke	High BP	Total (n)
# of events	3	18	5	4	28
% within group	3	15.0	13.5	10.3	

Summary of the two previous polymorphic patterns:

	Healthy	MI	Stroke	High BP	Total (n)
# of events	4	20	7	5	33
% within group	4	16.7	18.9	12.8	

AGT 620 C/T, AT1 1271 A/C, AT1 1167 A, AGR 395 T

	Healthy	MI	Stroke	High BP	Total (n)
# of events	2	8	1	2	13
% within group	2	6.7	2.7	5.1	

AGT 620 C/T, AT1 1271 A/C, AT1 1167 A/G, AGR 395 T

	Healthy	MI	Stroke	High BP	Total (n)
# of events	0	2	0	1	3
% within group	0	1.7	0	2.6	

AGT 620 C, AT1 1271 A, AT1 1167 A, AGR 395 A/T

	Healthy	MI	Stroke	High BP	Total (n)
# of events	1	4	5	3	11
% within group	1	3.3	13.5	7.7	

Summary of the three previous polymorphic patterns:

	Healthy	MI	Stroke	High BP	Total (n)
# of events	3	14	6	6	27
% within group	3	11.7	16.2	15.4	

AGT 620 C, AT1 678 A, AT1 1167 A, AGR 395 A/T

	Healthy	MI	Stroke	High BP	Total (n)
# of events	1	2	2	1	5
% within group	1	1.7	5.4	2.6	

AGT 620 C/T, AT1 678 C/T; AT1 1167 A, AGR 395 T

	Healthy	MI	Stroke	High BP	Total (n)
# of events	3	15	4	4	24
% within group	3	12.5	10.8	10.3	

Summary of the two previous polymorphic patterns:

	Healthy	MI	Stroke	High BP	Total (n)
# of events	4	17	6	5	29
% within group	4	14.2	16.2	12.9	

ACE 2193 A, AGR 1218 A, AGT 803 A

	Healthy	MI	Stroke	High BP	Total (n)
# of events	2	5	1	3	11
% within group	2	4.2	2.7	7.7	

ACE 2193 A, AGT 620 C/T

	Healthy	MI	Stroke	High BP	Total (n)
# of events	1	11	3	2	16
% within group	1	9.2	8.1	5.1	

ACE 2328 G, AGT 620 C/T

	Healthy	MI	Stroke	High BP	Total (n)
# of events	1	11	3	2	16
% within group	1	9.2	8.1	5.1	

ACE 3387 T, AGR 1204 A/C

	Healthy	MI	Stroke	High BP	Total (n)
# of events	0	10	3	3	15
% within group	0	8.3	8.1	7.7	

Example 3: Correlation Between a Specific Polymorphism Pattern and Treatment Response

The following study was undertaken to define polymorphic patterns in the human ACE, AGT, and/or AT1 genes that predict the efficacy of treatments for cardiovascular disease.

Two groups of hypertensive patients were studied, 41 in the first group and 20 in the second group. The groups were analyzed independently and in combination.

The patients in this population were each treated with one of the following five ACE inhibitors: Captopril, Trandolapril, Lisinopril, Fosinopril, or Enalapril. The effect of the drugs on mean arterial blood pressure was quantified. Mean arterial blood pressure was defined as 2/3 of the diastolic blood pressure + 1/3 of systolic blood pressure. The individuals were also categorized as "high responders," *i.e.*, those exhibiting a decrease of more than 16 mm Hg during treatment with an ACE inhibitor drug, and "low responders," *i.e.*, those not exhibiting a decrease of more than 16 mm Hg.

One particular polymorphic pattern, ACE 2193 A/G, AGR 1072 G/G, AT1 1167 A/A, which was present in 51% of the first study population, discriminated between high responders and low responders. In the second group of 20 patients, the pattern was

less prevalent (25%), but the correlation with lowered blood pressure remained statistically significant. Individuals having this polymorphic pattern (designated "1" below) experienced a larger decrease in blood pressure than those lacking this polymorphic pattern (designated "0" below).

Table 9 - Response to Treatment Correlates with Polymorphic Pattern

Polymorphic Pattern	Observations	Mean (mm Hg) Change in B.P.	S.D.
0	36	-11.4	8.6
1	25	-18.1	9.7

Furthermore, the distribution of high responders and low responders (as defined above) was as follows:

Table 10 - Responder Status Correlates with Polymorphic Pattern

Polymorphic Pattern	Low responder %	High responder %
0	80.1	19.4
1	24.0	76.0

Taken together, the results from the two groups indicate that the presence of this polymorphic pattern correlates with an incremental decrease of 6.4-7.3 mm Hg relative to individuals not having this polymorphic pattern.

The prevalence of this polymorphic pattern was 41% in this hypertensive population. This suggests that testing for this polymorphic pattern in hypertensive patients, followed by prescribing ACE inhibitors only to those patients having this polymorphic pattern, could increase the response rate from 43% (in a hypertensive population in general) to 76% in hypertensive population selected according to the methods of the invention.

If even one polymorphism was absent from the pattern, the high resolution of the variable and therefore the predictive value would be lost.

Example 4: Correlation Between a Specific Polymorphism Pattern and Treatment Response or Predisposition to a Cardiovascular Syndrome

In accordance with the methods disclosed in the above examples, additional correlations of polymorphic patterns with either responsiveness to ACE inhibitors, non-responsiveness to ACE inhibitors, predisposition to myocardial infarction, or predisposition to stroke were derived. These correlations are presented in the following table:

Table 11 - Additional Genetic Signatures Covering Components of the RAAS and Prediction

Genetic Signature	Position 1	Genotype1	Position 2	Genotype2	Position 3	Genotype3	Prediction:
GS1	ACE:2193:	A/G	AGR:1072:	G/G	AT1:1167:	A/A	Response to ACE-inhibitor treatment
GS2	ACE:2193:	A/G	AGR:1072:	G/G	ACE:1060:	G/G	Response to ACE-inhibitor treatment
GS3	ATR:2354:	A/A	AT1:678:	C/T	AT1:1167:	A/A	Response to ACE-inhibitor treatment
GS4	ACR:5496:	C/T	AGR:1204:	A/A	AGR:839:	G/G	Response to ACE-inhibitor treatment
GS5	ACR:5496:	C/T	AGR:1204:	A/A	AGT:620:	C/C	Response to ACE-inhibitor treatment
GS6	ACR:5496:	C/T	ACE:1060:	G/G	AGR:449:	C/T	Response to ACE-inhibitor treatment
GS7	ACR:5496:	C/T	ACE:1215:	C/T	AGR:1204:	A/A	Response to ACE-inhibitor treatment
GS9	ACR:5496	C/T	ACE:3906:	A/G	AGR:1218:	A/G	Response to ACE-inhibitor treatment
GS10	ACR:5496:	C/T	AGR:449:	C/T	AGR:839:	G/G	Response to ACE-inhibitor treatment
GS11	ACR:5496:	C/T	AGR:449:	C/T	AT1:1271:	A/A	Response to ACE-inhibitor treatment
GS12	ACR:5496:	C/T	AGR:449:	C/T	AGR:1072:	G/G	Response to ACE-inhibitor treatment
GS13	ACR:5496:	C/T	AGR:1072:	G/G	AGR:1204:	A/A	Response to ACE-inhibitor treatment
GS14	ACE:1060:	G/G	AGR:1204:	A/C	AGT:620:	C/C	Non-resposne to ACE-inhibitor treatment
GS15	ACE:1060:	G/G	AGR:449:	C/T	AGT:620:	C/C	Response to ACE-inhibitor treatment
GS16	ACE:1060:	G/G	AGR:1007:	G/G	AT1:678:	C/T	Response to ACE-inhibitor treatment
GS17	ACE:1215:	C/T	ACE:2193:	A/G	AGR:1204:	A/C	Non-response to ACE-inhibitor treatment
GS18	ACE:1215:	C/T	ACE:3906:	A/G	AGR:1218:	A/G	Response to ACE-inhibitor treatment

Genetic Signature	Position 1	Genotype1	Position 2	Genotype2	Position 3	Genotype3	Prediction:
GS19	ACE:2193:	A/G	ATR:2046:	C/T	AT1:678:	C/C	Non-response to ACE-inhibitor treatment
GS20	AGR:449:	C/T	AGR:1204:	A/A	AGT:620:	C/C	Response to ACE-inhibitor treatment
GS21	AGR:449:	C/T	AGR:1204:	A/A	AGT:1116:	G/G	Response to ACE-inhibitor treatment
GS22	AGR:449:	C/T	AGR:839:	G/G	AGT:620:	C/C	Response to ACE-inhibitor treatment
GS23	AGR:449:	C/T	AGT:620:	C/C	AGT:1116:	G/G	Response to ACE-inhibitor treatment
GS24	AGR:449:	C/T	AGT:1116:	G/G	AT1:1271:	A/A	Response to ACE-inhibitor treatment
GS25	AGR:449:	C/T	AGR:1072:	G/G	AGT:620:	C/C	Response to ACE-inhibitor treatment
GS26	AGR:1007:	G/C	AGR:1072:	G/G	AT1:678:	C/T	Response to ACE-inhibitor treatment
GS27	AGR:1072:	G/G	AGR:1204:	A/A	AGT:1116:	G/G	Response to ACE-inhibitor treatment
GS30	ACR:5496:	C/T	AGR:1204:	A/A	AT1:1167:	A/A	Response to ACE-inhibitor treatment
GS33	ACE:2193:	A/G	AT1:678:	C/C	AT1:1271:	A/C	Non-response to ACE-inhibitor treatment
GS36	AGR:1007:	G/G	AGR:1204:	A/A	AGT:1116:	G/G	Response to ACE-inhibitor treatment
GS37	ACE:2193:	A/A	AGR:1204:	A/C	-	-	Predisposition to MI
GS38	AGR:449:	T/T	AGR:1204:	A/C	AT1:1271:	A/C	Predisposition to MI
GS39	ACE:2193:	A/G	AGR:620:	C/T	AGR:1116:	G/G	Predisposition to MI
GS40	ACE:2193:	A/G	ANP:449:	T/T	AT1:1271:	A/C	Predisposition to MI
GS41	ACR:5349:	A/A	AGR:449:	T/T	AT1:1271:	A/C	Predisposition to MI
GS42	ACE:2193:	A/A	AGR:620:	C/T	AT1:1271:	A/A	Predisposition to MI
GS43	ACE:2193:	A/A	AGR:395:	A/T	-	-	Predisposition to stroke

Genetic Signature	Position 1	Genotype1	Position 2	Genotype2	Position 3	Genotype3	Prediction:
GS44	AGR:1007:	A/G	AGR:1072:	G/G	AT1:1167:	A/A	Predisposition to stroke
GS/45	AGR:395:	A/T	AGR:1072:	G/G	AGR:1218:	A/G	Predisposition to stroke

Polymorphic patterns, *i.e.*, combinations of sequences at particular polymorphic positions, that show a statistically significant correlation with myocardial infarction and stroke are shown below:

Table 12 - Correlation with MI

MI (n=120)

Healthy (n=100)

Genetic Signature	Prevalence	MI	Healthy	P-value
ACE:2193 AA ANP:1204 AC	5.0%	100%	0%	0.001
AGR:449 TT AGR:1204 AC AT1:1271 AC	8.2%	89%	11%	0.002
ACE:2193 AG AGT:620 CT AGT:1167 GG	4.5%	100%	0%	0.002
ACE:2193 AG AGR:449 TT AT1:1271 AC	17.7%	74%	26%	0.007
ACR:5349 AA AGR:449 TT AT1:1271 AC	14.5%	75%	25%	0.013
ACE:2193 AA AGT:620 CT AT1:1271 AA	3.6%	100%	0%	0.009

Table 13 - Correlation With Stroke

Stroke (n=37)
Healthy (n=100)

Genetic Signature	Prevalence	Stroke	Healthy	P-value
ACE:2193 AA AGR:395 AT	2.9%	100%	0%	0.005
AGR:1007 AG AGR:1072 GG AT1:1167 AA	9.5%	85%	15%	0.000008
AGR:395 AT AGR:1072 GG AGR:1218 AG	4.4%	83%	17%	0.006

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description.

Various patents, patent applications, methodologies, and publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

Example 5: Correlation Between a Specific Polymorphism Pattern and High Blood Pressure Treatment with a β-Blocker

Using the techniques described in Examples 1 and 2, we investigated polymorphisms in the ADBP1 and ADBR2 genes (regulator/promoter regions and coding regions). The source material was the same as described above.

At the outset, we identified a set of key polymorphisms. Some of these are known; others are new. The are summarized in Table 14.

Table 14 -Polymorphic Positions in the Genes Encoding the Beta Adrenergic Receptors 1 and 2

Gene:Location	Genotypes		
B1P:2238	A/A	A/G	G/G
B1P:2577	C/C	C/T	T/T
B1P:2757	A/A	A/G	G/G
B1R:231	A/A	A/G	G/G
B1R:758	C/C	C/T	T/T
B1R:1251	C/C	C/G	G/G
B1R:1403	A/A	A/G	G/G
B1R:1528	A/A	A/C	C/C
B2P:934	A/A	A/G	G/G
B2P:987	C/C	C/G	G/G
B2P:1006	A/A	A/G	G/G
B2P:1120	C/C	C/G	G/G
B2P:1221	C/C	C/T	T/T
B2P:1541	C/C	C/T	T/T
B2P:1568	C/C	C/T	T/T
B2R:839	A/A	A/G	G/G
B2R:872	C/C	C/G	G/G
B2R:1045	A/A	A/G	G/G
B2R:1284	C/C	C/T	T/T
B2R:1316	A/A	A/C	C/C
B2R:1846	C/C	C/G	G/G
B2R:2032	A/A	A/G	G/G
B2R:2068	no insertion	insertion G/G	insertion C/G
B2R:2070	no insertion	insertion C/C	

Abbreviations used for the denotation of the genes:

B1P (ADBR1) - putative promoter region of the gene encoding the beta adrenergic receptor 1

B1R (ADBR1) - the protein coding region of the gene encoding the beta adrenergic receptor 1

B2P (ADBR2) - putative promoter region of the gene encoding the beta adrenergic receptor 2

B2R (ADBR2) - the protein coding region of the gene encoding the beta adrenergic receptor 2

These positions, one or a combination of several, can be used to predict a good response to anti-hypertensive treatment with Beta-blockers.

Indeed, we identified various polymorphisms/polymorphic patterns that can be used in the prediction of high response to anti-hypertensive treatment with beta-blockers. These are shown in Table 15.

Table 15 - Polymorphisms Predictive of β-Blocker Responsitivity

GS	Positions - genotypes	Prevalence (%)	P-value	Comments
GS1	B2R:1846 - C/C	7.7	0.0049	Patients show a 8.3 mmHg DBP reduction compared to the group lacking this genotype.
GS2	B2R:1045 - A/A	6.6	0.0105	Patients will show a 8.1 mmHg DBP reduction compared to the group lacking this genotype.
GS3	B2R:1316 - A/A	6.6	0.0105	Patients that will show a 8.1 mmHg DBP reduction compared to the group lacking this genotype.

Not only positions in the beta adrenergic receptors, but also positions in the renin-angiotensin- aldosterone-system can be used to predict response to anti-hypertensive treatment with beta- blockers (*see* Table 16). The numbering give according to the numberings in GenBank as defined in Table 1, *supra*.

Table 16 - RAAS Polymorphism Associated with β-Blocker Responsitivity

GS	Positions - Genotypes	Prevalence (%)	Comments
GS105B	ATR:2046 - C/C + AT1:678 - T/T	10	Patients will show more that 10-mmHg DBP (diastolic blood pressure reduction compared to the group lacking this genotype.

* * *

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

It is further to be understood that all sizes and all molecular weight or molecular mass values are approximate, and are provided for description.

Patents, patent applications, procedures, and publications cited throughout this application are incorporated herein by reference in their entireties.

WHAT IS CLAIMED IS:

1. A method for assessing cardiovascular status in an individual to be tested comprising comparing

(a) a test polymorphic pattern comprising at least one polymorphic position within a gene encoding a polypeptide selected from the group consisting of renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, and β-adrenoceptor of the individual, with

(b) a reference polymorphic pattern derived from a population of individuals exhibiting a predetermined cardiovascular status; and

concluding whether the individual possesses the cardiovascular status based on whether the test pattern matches the reference pattern.

2. The method according to claim 1, wherein the polymorphic pattern of (a) includes a polymorphic position within a second gene encoding a polypeptide selected from the group consisting of ACE, AT1, and AGT.

3. The method according to claims 1 or 2, wherein the predetermined cardiovascular status is predisposition to a cardiovascular syndrome.

4. The method according to claim 3, wherein the cardiovascular syndrome is selected from the group consisting of myocardial infarction, unstable angina, hypertension, atherosclerosis, and stroke.

5. The method according to claim 3, wherein the reference pattern comprises at least two polymorphisms.

6. The method according to claim 5, wherein the reference pattern comprises at least three polymorphisms.

7. The method according to claims 1 or 2, wherein the predetermined cardiovascular status is a response to a cardiovascular treatment regimen.

8. The method according to claim 7, wherein the treatment regimen comprises administering a cardiovascular drug selected from the group consisting of an ACE inhibitor, an angiotensin II receptor antagonist, a diuretic, an alpha-adrenoreceptor antagonist, a cardiac glycoside, a phosphodiesterase inhibitor, a beta-adrenoreceptor antagonist, a calcium channel blocker, a HMG-CoA reductase inhibitor, an imidizoline receptor blocker, an endothelin receptor blocker, and an organic nitrite.

9. The method according to claim 1, wherein the polymorphic position of the gene is selected from the group consisting of a position in the renin coding region comprising a cytosine to thymine transition in exon 10 that creates a premature stop codon at position 387 resulting in a truncated form of renin with 20 amino acids deleted from the carboxyl terminus; a position in a promoter region of aldosterone synthase numbered -344 (with the initiation codon starting at 1); positions in the regulatory region of β-adrenergic receptor-1 gene (designated B1P) numbered 2238, 2440, 2493, 2502, 2577, 2585, 2693, 2724, and 2757; positions in the β-adrenergic receptor-1 coding region (designated B1R) numbered 231, 758,1037, 1251, 1403, and 1528; positions in the β-adrenergic receptor-2 gene regulatory region (designated B2P) numbered 932, 934, 987, 1006, 1120, 1221, 1541, and 1568; positions in the β-adrenergic receptor-2 gene coding region (designated B2R) numbered 839, 872, 1045, 1284, 1316, 1846, 1891, 2032, 2068, and 2070; positions in the endothelin receptor type A coding region (designated ET_A) numbered 969, 1005, 1146, and 2485; and combinations of any of the foregoing.

10. The method according to claim 2, wherein the polymorphic position of the second gene is selected from the group consisting of positions in the ACE regulatory region numbered 5106, 5349, and 5496; positions in the ACE coding region numbered 375, 582, 731, 1060, 1215, 2193, 2328, 2741, 3132, 3387, 3503, and 3906; position 1451 in the ACE gene as numbered in GenBank entry X62855; positions in the AGT regulatory region numbered 395, 412, 432, 449, 692, 839, 1007, 1072, 1204, and 1218; positions in the AGT coding region numbered 273, 620, 803, 912, 997, 1116, and 1174; position 49 in the AGT gene as numbered in GenBank entry M24688; positions in the AT1 regulatory region numbered 1427, 1756, 1853, 2046, 2354, 2355, and 2415; and positions in the AT1 coding region numbered 449, 678, 1167, and 1271.

11. An isolated nucleic acid encoding a polypeptide selected from the group consisting of renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, and β-adrenoceptor in an individual, wherein the nucleic acid comprises a polymorphic position and wherein the polymorphic position is a position in a polymorphic pattern derived from a population of individuals exhibiting a predetermined cardiovascular status.

12. A probe which hybridizes at high stringency to a polymorphic position as defined in claim 11.

13. A library of nucleic acids comprising a nucleic acid which comprises

(a) a polymorphic position of a gene encoding a polypeptide selected from the group consisting of renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, and β-adrenoceptor, wherein the polymorphic position is a position in a polymorphic pattern derived from a population of individuals exhibiting a predetermined cardiovascular status, and

(b) nucleic acids comprising other polymorphic positions of human genes that are indicative of cardiovascular status of an individual, wherein the human genes are selected from the group consisting of ACE, AT1, AGT, renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, and β-adrenoceptor.

14. The library of claim 13 wherein the nucleic acids are oligonucleotides.

15. A kit for assessing cardiovascular status comprising

(a) sequence determination primers and

(b) sequence determination reagents,

wherein the primers are selected from the group consisting of primers that hybridize to or immediately adjacent to

(i) a polymorphic position in a gene gene encoding a polypeptide selected from the group consisting of renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, and β-adrenoceptor, and

(ii) a second polymorphic position in a gene encoding a polypeptide selected from the group consisting of ACE, AT1, AGT, renin, aldosterone synthase, type-2 angiotensin II receptor, endothelin receptor, and β-adrenoceptor.

16. The kit of claim 15, wherein the second polymorphic position is selected from the group consisting of positions in the ACE regulatory region numbered 5106, 5349, and 5496; positions in the ACE coding region numbered 375, 582, 731, 1060, 1215, 2193, 2328, 2741, 3132, 3387, 3503, and 3906; position 1451 in the ACE gene as numbered in GenBank entry X62855; positions in the AGT regulatory region numbered 395, 412, 432, 449, 692, 839, 1007, 1072, 1204, and 1218; positions in the AGT coding region numbered 273, 620, 803, 912, 997, 1116, and 1174; position 49 in the AGT gene as numbered in GenBank entry M24688; positions in the AT1 regulatory region numbered 1427, 1756, 1853, 2046, 2354, 2355, and 2415; positions in the AT1 coding region numbered 449, 678, 1167, and 1271; a position in the renin coding region comprising a cytosine to thymine transition in exon 10 that creates a premature stop codon at position 387 resulting in a truncated form of renin with 20 amino acids deleted from the carboxyl terminus; a position in a promoter region of aldosterone synthase numbered -344 (with the initiation codon starting at 1); positions in the regulatory region of β-adrenergic receptor-1 gene (designated B1P) numbered 2238, 2440, 2493, 2502, 2577, 2585, 2693, 2724, and 2757; positions in the β-adrenergic receptor-1 gene coding region (designated B1R) numbered 231, 758, 1037, 1251, 1403, and 1528; positions in the β-adrenergic receptor-2 regulatory region (designated B2P) numbered 932, 934, 987, 1006, 1120, 1221, 1541, and 1568; positions in the β-adrenergic receptor-2 gene coding region (designated B2R) numbered 839, 872, 1045, 1284, 1316, 1846, 1891, 2032, 2068, and 2070; positions in the endothelin receptor type A coding region (designated ET_A) numbered 969, 1005, 1146, and 2485; and combinations of any of the foregoing.

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Andersson, Maria Kristina
Lindstrom, Per Harry Rutger

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(71) Applicant *(for all designated States except US)*: EURONA MEDICAL AB [SE/SE]; Kungsgangsvägen 29, S–751 06 Uppsala (SE).

(72) Inventors; and

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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14 September 2000 (14.09.00)

(54) Title: GENES FOR ASSESSING CARDIOVASCULAR STATUS AND COMPOSITIONS FOR USE THEREOF

(57) Abstract

The present invention provides methods for assessing cardiovascular status in an individual, which comprise determining the sequence at one or more polymorphic positions within the human genes encoding ACE, AT1, AGT, renin, aldosterone synthase, type–2 angiotensin II receptor, endothelin receptor, and β–adrenoceptor. The invention also provides isolated nucleic acids encoding polymorphisms in genes encoding ACE, AT1, AGT, renin, aldosterone synthase, type–2 angiotensin II receptor, endothelin receptor and β–adrenoceptor, nucleic acid probes that hybridized to polymorphic positions, kits for the prediction of cardiovascular status, and nucleic acid and peptide targets for use in identifying candidate cardiovascular drugs.

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INTERNATIONAL SEARCH REPORT

Inter. nal Application No
PCT/IB 99/01678

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68 C07K14/705 C12N9/64 C07K14/72 C12N9/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

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IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	WO 87 02709 A (BIOTECH RES PARTNERS LTD) 7 May 1987 (1987-05-07) the whole document	1,3,4, 11,12

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
10 May 2000	22/05/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Molina Galan, E

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INTERNATIONAL SEARCH REPORT

Inter. nal Application No
PCT/IB 99/01678

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INTERNATIONAL SEARCH REPORT

information on patent family members

Intern nal Application No
PCT/IB 99/01678

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